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Developing molecular tools to assess the biogeochemical/microbial community structure of oil sand processed waste material

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By

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Developing molecular tools to assess the biogeochemical/microbial community structure of oil sand processed waste material

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DECLARATION OF CO-AUTHORSHIP AND PREVIOUS PUBLICATION

I. Co-authorship Declaration

I hereby declare that this thesis incorporates material that is the result of joint research, as follows:

This thesis incorporates research undertaken in collaboration with N. Loick under the supervision of C. Weisener. This collaboration is covered in Chapters 2 and 3 of this thesis. In all cases, the key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of the co-authors was primarily through the assistance in lab work and comments on the manuscript.

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Chapter 2	Assessment of RNA preservation methods to study active microbial population in oil sands tailings ponds	In preparation

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ABSTRACT

Microbial communities can dominate Fluid Fine Tailings (FFT) in the presence of electron acceptors (e.g. Sulfate). Sulfate reduction can produce hydrogen sulfide, one of several chemical constituents responsible for sediment oxygen demand (SOD). The preservation of RNA is a crucial step to study active microbial populations and their activity in FFT and hence understand the biological factors contributing to SOD. In our study different RNA preservation methods were tested to preserve microbial RNA in FFT sample. The results confirmed that LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc, California) is the best preservative method for RNA preservation. Through T-RFLP analysis of 16s rRNA and 16s rDNA, SRB's (Sulfate Reducing Bacteria) are shown to dominate the FFT during initial stages of incubation but its population decreased significantly over-time. This observation suggests that sulfate reduction is a self-limiting process and has less impact on the quality of overlying water column.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DNA - Deoxyribonucleic acid

RNA - Ribonucleic acid

rRNA - Ribosomal ribonucleic acid

mRNA - Messenger ribonucleic acid

T-RFLP - Terminal Restriction Fragment Length Polymorphism

TRF - Terminal Restriction Fragment

SOD - Sediment oxygen demand

DO – Dissolved Oxygen

FFT – Fluid Fine Tailings

SRB - Sulfate-reducing bacteria

PAT - Phylogenetic Assignment Tool

PCA - Principal Component Analysis

PMA - Propidium Monoazide

Chapter 1- Introduction

1.1 Alberta oil sands

Oil sands deposits have been discovered around the world and include Canada, Kazakhstan and Russia. Northern Alberta, Canada has the largest deposit of approximately 2.5 trillion barrels of recoverable bitumen held in a mineral matrix of sand, clay and water (Fig 1.1) (Penner and Foght. 2010). Currently nearly 1.31 million barrels of bitumen are extracted every day and this is expected to increase to roughly 3 million barrels per day by 2018 (Alberta energy. 2013). Based on the location of oil sands, two different methods are used in bitumen recovery. If the deposit is shallow the surface mining method is used. In case of deeper deposits, in situ recovery methods like cyclic steam stimulator and steam assisted gravity drainage are used (Li. 2010). At present, open pit surface mining is the method widely employed for oil sands extraction. After mining the bitumen is separated out of oil sands by the Clark hot water extraction process. In this process, crushed oil sands are treated with Caustic hot water (50 – 80ºC) to reduce the viscosity of bitumen and the flotation technique is used to recover bitumen in the form of bitumen froth (Chalaturnyk et al. 2002). The sands separated during this process are utilized in the construction of tailings ponds.

1.2 Oil sands tailings and reclamation

The processing of oil sands to produce synthetic crude oil generates a large volume of tailings (Fig 1.2). The tailings are mainly composed of water, sand, fines (clay <44µm), residual bitumen (0.5%-5% mass) and naphtha (<0.5%) (Chalaturnyk et al. 2002). In order to produce one barrel of bitumen about 1 m^3 of oil sands and 3 m^3 of water are used. This process results in 4 m^3 of tailings per barrel of extracted bitumen (Holowenko et al. 2000). On average about 262,000 m^3 of tailings are produced per day. The oil sands companies operate under a zero discharge policy; therefore the tailings are stored on site and are kept in settling basins generally called “oil sands tailings ponds” (Fig 1.3) (Fedorak et al. 2002).

The tailing ponds contain approximately 840 million m^3 of fine tailings that cover roughly 170 km^2 of oil sands region (Siddique et al. 2011). As the tailings are allowed to settle in tailings ponds, the sand particles settle quickly to the bottom to form a base. The remaining tailings take years to densify forming thick slurry called Fluid Fine Tailings (FFT) (Penner and Foght. 2010). As a measure to reduce the size of the tailings ponds and to reduce the fresh water usage, the overlaying water released from the tailings ponds are reused for oil sands processing. The FFT after 10-15 years of densification will be transferred into the mined-out pits. These mined-out pits will be capped with a large amount of fresh water to form end-pit lakes (EPL) (Zubot. 2010). These lakes will be organized in such a way to support all life forms. This process is called "Wet landscape approach" one of the proposed reclamation methods for oil sands tailings.

1.3 Wetlands ecosystem and sediment oxygen demand

Wetlands are well known to provide habitat for many plants and animals creating an important site for cycling of key nutrients like carbon, nitrogen, sulfur and phosphorus (Batzer and Sharitz. 2006). Dissolved oxygen, being a vital component for many of the organisms, will decide the functioning of wetlands (Dauer et al. 1992). The biogeochemical processes taking place at the water-sediment interface leads to the consumption of dissolved oxygen causing Sediment Oxygen Demand (SOD) (Murphy and Hicks. 1986). SOD is the major contributor to oxygen depletion in water bodies and it is affected by factors like temperature, chemical component and dissolved oxygen content. SOD is composed of two major components, the biological sediment oxygen demand (BOD) and chemical sediment oxygen demand (COD) (Wang and Paula. 1984). In all wetlands the composition of sediment plays a key role in its functionality. Therefore the study of sediment is crucial to conserve the wetlands. In case of oil sands tailings ponds, during reclamation the wetlands will be developed on top of FFT. Therefore the study on biogeochemical process in FFT is crucial for the success of the wetland ecosystems.

1.4 Role of microorganisms in wetlands

Microorganisms are the driving force of biogeochemical processes in wetlands, the study of microbial communities are essential for the success of a wetland ecosystem. The FFT material is complex in nature and has the ability to support a wide range of biochemical process which have a direct impact on the life forms inhabiting the overlying water column. This study will focus on the biochemical processes in FFT and their role on sediment oxygen demand in the overlying water column. In the aerobic zone (presence of oxygen) microorganisms dwelling in the water-sediment interface will consume oxygen by degrading organic compounds leading to the BOD. The FFT is an organic rich and viscous material; anaerobic reactions will be the most prevalent processes in FFT. The oxygen diffusion to the FFT is limited because of overlaying water columns and the existing dissolved oxygen is also quickly utilized by microbes during hydrocarbon degradation. Iron reduction, sulfate reduction and methanogenesis are some of the major processes under anaerobic conditions. The anaerobic microorganisms produce reduced chemical species like hydrogen sulfide and methane (Fig 1.4). Reduced chemical substances in the sediments reaching the overlying water column will get re-oxidized leading to COD (Gelda et al. 1995). This study focuses on sulfate reduction, which is one of the major anaerobic processes as the hydrogen sulfide produced during this process is a potent reducing agent and is toxic to aquatic organisms (Smith and Oseid. 1971). Hydrogen sulfide has the ability to form metal sulfides especially iron sulfide (Schoonen, 2004). With iron being an essential nutrient for microorganisms (Church et al 2000), the iron sulfide formation may affect the growth of other useful microorganisms. The predominant presence of SRB's and methanogens in tailings ponds has been reported in previous studies (Holowenko et al. 2000, Siddique et al. 2006, Ramos-Padrón E et al., 2011). However, long-term assessment of SRB activity is still lacking and is crucial for the success of reclamation of tailings ponds.

1.5 Study of active microbial population

Several studies have reported the role of microorganisms in tailings ponds. Most of these studies were performed using DNA (Chi Fru et al., 2013, Penner and Foght. 2010, Holowenko et al. 2000, Siddique et al. 2006, Ramos-Padrón E et al., 2011). DNA, being a stable

macromolecule, is known to persist in soil for a long time. DNA isolated from the soil samples will be comprised of extracellular DNA and intracellular DNA from live and dead cells. As a result, DNA analysis may lead to overestimation of microbial populations (Josephson et al. 1993, Masters et al. 1994). Propidium Monoazide (PMA), a DNA-binding dye has been reported to solve this problem (Nocker et al., 2006, 2007). PMA has the ability to bind to double-stranded DNA and block its amplification during PCR amplification by forming a stable covalent nitrogen-carbon bond. Upon photo activation, the azide group of the dye is converted into a nitrene radical reacting with any hydrocarbon moiety in the binding site, leading to a permanent modification of the DNA. The peculiarity of the dye is its ability to penetrate only the cell membrane of dead cells. Therefore on treating samples with PMA only the DNA from live cells will get amplified during PCR (Taskin et al. 2011).

Even though PMA treated DNA (PMA-DNA) can provide information about the live microbial community, it is not able to differentiate between active and dormant microorganisms (Nocker et al. 2007). Therefore to track the active microbial population RNA can be utilized. RNA is a highly unstable macromolecule (Deutscher 2006) and is produced mostly while the cells are active (Fig 1.5). There are two different types of RNA widely used in the field of microbial ecology, these are rRNA and mRNA. The 16s rRNA is the central component in protein synthesis, widely used to track the active microbial community structure because of its unique properties; such as universal distribution, high conservation, considerable variability and minimal lateral gene transfer. The mRNA is related to gene expression and protein synthesis and can be used to study microbial activity in order to understand chemical processes like sulfate reduction (Strattan. 2010; Farrell. 2011). Even though the RNA analysis is an effective method to study the active microbial population, the unstable nature of RNA leads to significant losses during sample collection. This is true especially in case of complex environmental samples like FFT where the presence of humic acid and other components may have an impact on RNA quality. Therefore, developing a microbial soil RNA preservation method compatible with the FFT is crucial to understand the microbial structure and activity. In this study, four different preservation methods were tested using T-RFLP analysis (details of the methods in chapter2) in order to find the best preservation method for FFT. The compatible RNA preservation method will find its application in the field FFT sample collection and preservation. This preservation is

essential to prevent the degradation of RNA during sample shipping. The RNA analysis of preserved FFT samples can provide uncompromised information about the microbial community structure and activity in tailings ponds during the time of sample collection. The information on microbial population is vital for pond management and for the development of reclamation methods.

1.6 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP is a PCR-based tool widely used to monitor changes in the structure and composition of microbial communities (Clement et al. 1998, Liu et al. 1997, Dunbar et al. 2000, Wu et al. 2006, Ramakrishnan et al. 2000). During T-RFLP analysis the target gene will be amplified by PCR, wherein one or both the primers will be labelled with a fluorescent dye. These PCR amplicons will be subjected to restriction digestion using one or more restriction enzymes (Four base pair recognition sites). As different species will have different 16s rRNA gene sequences, the length of the TRF (Terminal Restriction Fragment) generated after restriction digestion can be directly related to a particular species. The size and relative abundance of the TRFs are determined using an automated DNA sequencer (Schütte et al. 2008). The T-RFLP data can be employed for two purposes, first to determine changes in microbial community structure through statistical analysis and second to perform species identification through web-based tools like Phylogenetic Assignment Tool (PAT) (Kent et al., 2003). Even though the T-RFLP faces the problems associated with any PCR-based method such as formation of chimerical and heteroduplex molecules (Acinas et al. 2005, Becker et al. 2000), the simplicity of the method makes it the most preferred method in microbial ecology.

1.7 Laboratory microcosm studies

Laboratory microcosms are widely used to simulate the behavior of natural ecosystems under controlled conditions (Fig 1.6). The microcosms are easy to handle and the sample collection from microcosms can be performed at regular intervals making it an ideal choice for long-term assessments (Jessup et al, 2004). In case of oil sands tailings ponds, the field studies are performed with great difficulty because of extreme cold conditions (-54°C) and other safety

requirements. Therefore the long-term analysis performed using microcosms can be used to understand microbial community structure of tailings ponds.

A study conducted by Chi Fru et al. 2013 proved that the microbial community structure stimulated through microcosm/bioreactor studies is similar to field samples. In the study the T-RFLP analysis of 16s rDNA was performed to study bacterial and archaeal community structure in FFT and their change over time. Through the analysis it was found that the FFT was initially dominated by bacterial population and later by archaeal population. This similar pattern was observed in field studies, where the development of sulfide rich zones at the sediment - water interface proved the dominance of bacterial population especially SRB. After the decline in SRB population, the emergences of methanogens were detected through methane bubbling and MPN techniques.

Figure 1.1: Alberta oil sands deposits. Map taken from Alberta energy (www.energy.alberta.ca)

Figure 1.2: Oil Sands Processed Material

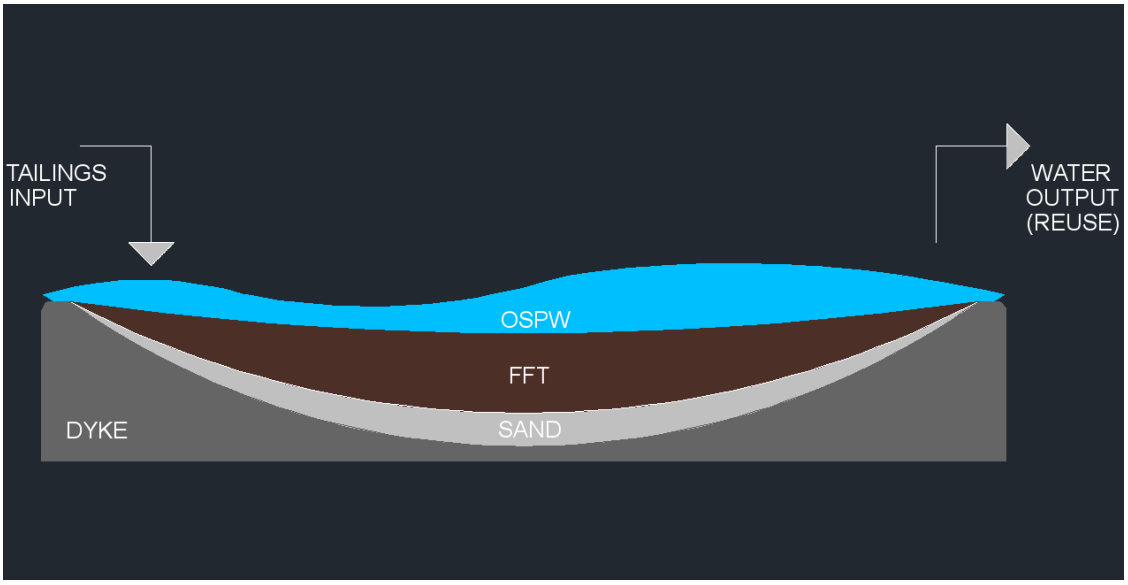

Figure 1.3: Conceptual diagram – Oil sands tailings ponds (FFT-Fluid Fine Tailings, OSPW – Oil Sands Processed Water)

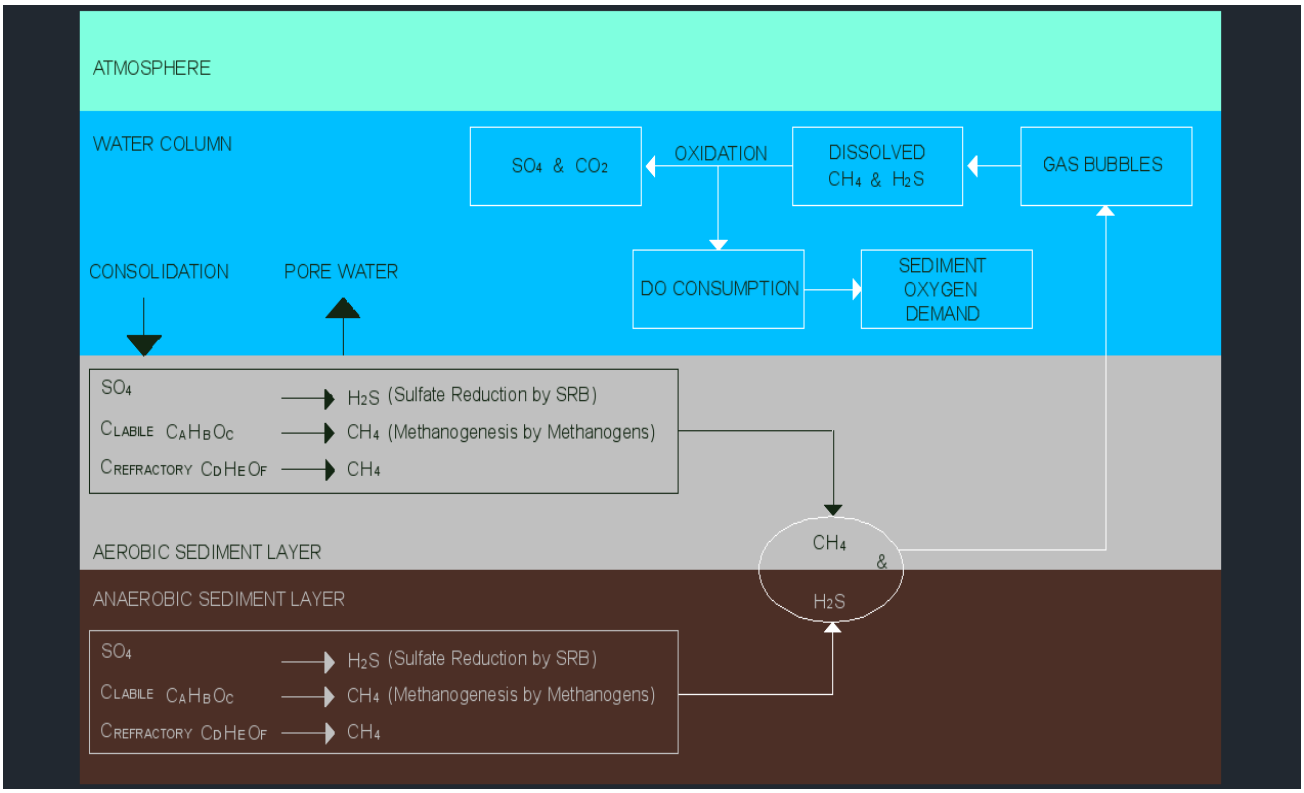

Figure 1.4: Conceptual diagram to depict the use of SRB and Methanogens for Chemical Sediment Oxygen Demand (CSOD) in wetlands

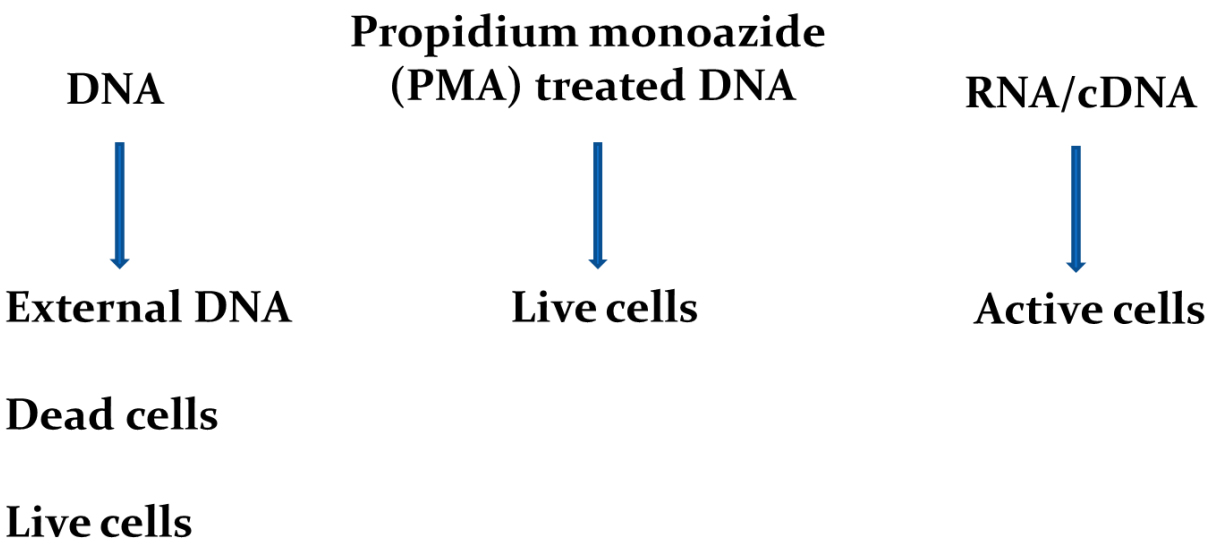

Figure 1.5: Flow Chart to compare DNA, PMA-DNA and RNA based on their role in microbial ecology

Figure 1.6: Laboratory microcosm

1.8 Hypothesis and objectives:

The LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc, California) has been specifically designed for RNA preservation in soil samples, but it is unknown whether this off the counter product can be used to preserve RNA in active oil sands process materials (OSPM). In this study (Chapter 2) it is hypothesized that LifeGuard™ will preserve the expressed RNA more efficiently than other methods like RNAlater method, Glycerol method and flash freezing. The LifeGuard™ soil preservation solution is more efficient because of its biostatic activity and ability to inactivate RNase in soil. Therefore it is expected to be effective in both long-term and short-term storage of soil microbial RNA compared to the other methods which lack one or both properties. If this hypothesis holds true, the bacterial community structure of both control and LifeGuard treated samples should be similar. They should establish a closer relationship with each other during the statistical analysis. The RNA extraction in control samples are performed immediately after sample collection, thus the RNA degradation will be negligible, therefore the control sample can reflect the actual bacterial community structure of FFT. To test the suitability and effectiveness of LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc, California) RNA will be extracted from differently preserved tailings samples and T-RFLP analysis of 16s rRNA/cDNA will be performed to compare the bacterial community structure. Once the RNA protocol has been tested and optimized it will be validated using laboratory microcosm.

In Chapter 3, the statistical analysis of the TRFLP data for bacterial 16s rDNA (PMA-DNA) will be performed to determine the similarity/difference between the FFT samples collected at different depth and different atmospheric conditions. It is hypothesized that the population of sulfate reducing bacteria will dominate and then decrease as the system matures in part due to the decrease in sulfate concentration and hydrogen sulfide production, which is toxic to bacteria. If this hypothesis holds true, the statistical analysis of T-RFLP data is expected show a higher bacterial population and the species identification should show different SRB species during the initial stages of development. As the system matures, less or no SRB species should be detected in FFT. To test this hypothesis, species identification will be performed using PAT in order to get a general idea about the bacterial population present in the FFT and to track the presence/absence of sulfate reducing bacteria.

CHAPTER 2 - Assessment of RNA preservation methods to study active microbial population in oil sands tailings ponds

2.1 Introduction

The aim of the project being the long-term assessment of microbial populations, the utilization of RNA is crucial to track only the active microbial population and not dead cells. Even though the RNA has a major role in microbial ecology, unlike DNA, the RNA is highly unstable which poses a major problem of RNA degradation within a few minutes to hours of cell death. Therefore developing a preservation method is indispensable for any further RNA analysis to be meaningful. Flash freezing is the most widely used RNA preservative method. Even though flash freezing has been successfully applied for soil RNA preservation (Rissanen et al. 2010, Wallenius et al. 2010) liquid nitrogen is not always accessible, especially in the case of oil sands tailings a lot of restrictions apply because of on-site health and safety issues. Therefore finding an alternative method is crucial for soil RNA preservation.

In this study four different preservation methods are investigated.

1. LifeGuard™ Soil Preservation Solution
2. RNAlater® solution
3. Glycerol
4. Liquid Nitrogen

The LifeGuard™ Soil Preservation Solution is supplied by MO BIO Laboratories, Inc, California and specially formulated for soil samples. The RNAlater® solution is supplied by Life Technologies Corporation and is formulated for tissue samples but has been widely used in studies for soil RNA preservation (Foti et al., 2008). The manufacturer gives an indefinite storage time for RNA in this solution, which is important for long-term projects. Glycerol is selected in this study because it is a very cost effective and commonly used chemical in the laboratory and has been proven to be effective in the preservation of microorganisms and soil samples (Sessitsch et al. 2002) but its impact on complex environmental samples like tailings needs to be tested. Liquid nitrogen is widely tested with all kinds of environmental samples

including mineral soil, compost, sediments. This method can be used along with a control to validate other methods.

In this study T-RFLP of 16s rRNA/cDNA was used to compare the microbial community profiles detected with the different preservation methods. Different statistical methods were used to compare the community profiles with the objective to detect the preservation method with higher similarity to the control sample. The method with the highest similarity will be declared the best soil RNA preservation method. The comparison between RNA, DNA and PMA-DNA were also performed based on T-RFLP data to determine their role in microbial ecology.

2.2 Materials and Methods

2.2.1 Sample collection and Preservation

The samples used in this experiment were oil sands tailings. The samples were collected from a microcosm (details in chapter 3 methods), which was maintained to perform other laboratory studies. The microcosm was 20 weeks old during the time of sample collection. Immediately after sample collection, approximately 3 g of samples was transferred to 50 ml sterile centrifuge tubes and was treated with different preservative solutions (Fig 2.1). In case of control sample immediately after sample collection the RNA extraction was performed.

The following preservation method was applied

i. Lifeguard method: In case of Lifeguard preservative method about 9 ml of LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc, California) was added to 3 g of samples and mixed thoroughly according to manufacturer's instruction. The samples were stored in -80°C for a period of 3 & 30 days until the extraction of RNA.

ii. RNAlater® method: About 15 ml of RNAlater® solution (Life Technologies Corporation) was added to 3 g of samples and mixed thoroughly according to manufacturer's instruction. The samples were stored overnight at 4°C and then stored in -80°C for a period of 3 & 30 days until the extraction of RNA.

iii. Glycerol Method: About 9 ml of glycerol (15% glycerol, 0.85% NaCl) was added to 3 g of samples and mixed thoroughly (Sessitsch et al. 2002). The samples were stored in -80°C for a period of 3 & 30 days until the extraction of RNA.

iv. Liquid Nitrogen: The samples were frozen immediately using liquid nitrogen and were stored in -80°C for a period of 3 & 30 days until the extraction of RNA.

2.2.2 RNA extraction, DNA digestion and cDNA synthesis

After 3 and 30 days (Short-term and Long-term) of incubation the RNA extraction was performed using RNA PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories, Inc, California) following manufacturer's instructions. The extracted RNA was stored at -80°C. After RNA extraction, the RNA capture columns were used to co-elute DNA using PowerSoil® DNA Elution Accessory Kit (MO BIO Laboratories, Inc, California). The extracted EDNA (Eluted DNA) was stored at -20°C. The extractions were performed in triplicate and were pooled together before performing further experiments. In case of RNA, in order to perform DNA digestion 12.5 µl of RNA sample was added with 1 µl of DNase enzyme and 1.5 µl DNase buffer (Ambion Inc.) and digestion was performed according to manufacturer's instruction. For the digested sample about 10 µl of the sample was used for CDNA synthesis. The cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®) according to manufacturer's instruction.

2.2.3 PMA treatment and DNA extraction

PMA™ (Propidium Monoazide) dye, 20 mM in H_2O (Biotium, Inc) was used in the experiment. Immediately after sample collection, 0.25 g of the samples was treated with 100 µM of PMA and mixed thoroughly. The samples were incubated in the dark for 5 minutes at room temperature. After incubation the sample tubes were placed on the ice and were exposed to light for 5 minutes using 600 w halogen light source. Immediately after PMA treatment the DNA extractions were performed using PowerSoil® DNA Isolation Kit (Mo Bio, Laboratories Inc, California) following manufacturer's instructions. In case of PMA untreated sample the samples were stored at -20°C and the extractions were done after one week using PowerSoil® DNA

Isolation Kit (Mo Bio, Laboratories Inc, California) following manufacturer's instructions. The extracted DNA was stored at -20°C (Fig 2.1). The extractions were performed in triplicate and were pooled together before performing further experiments.

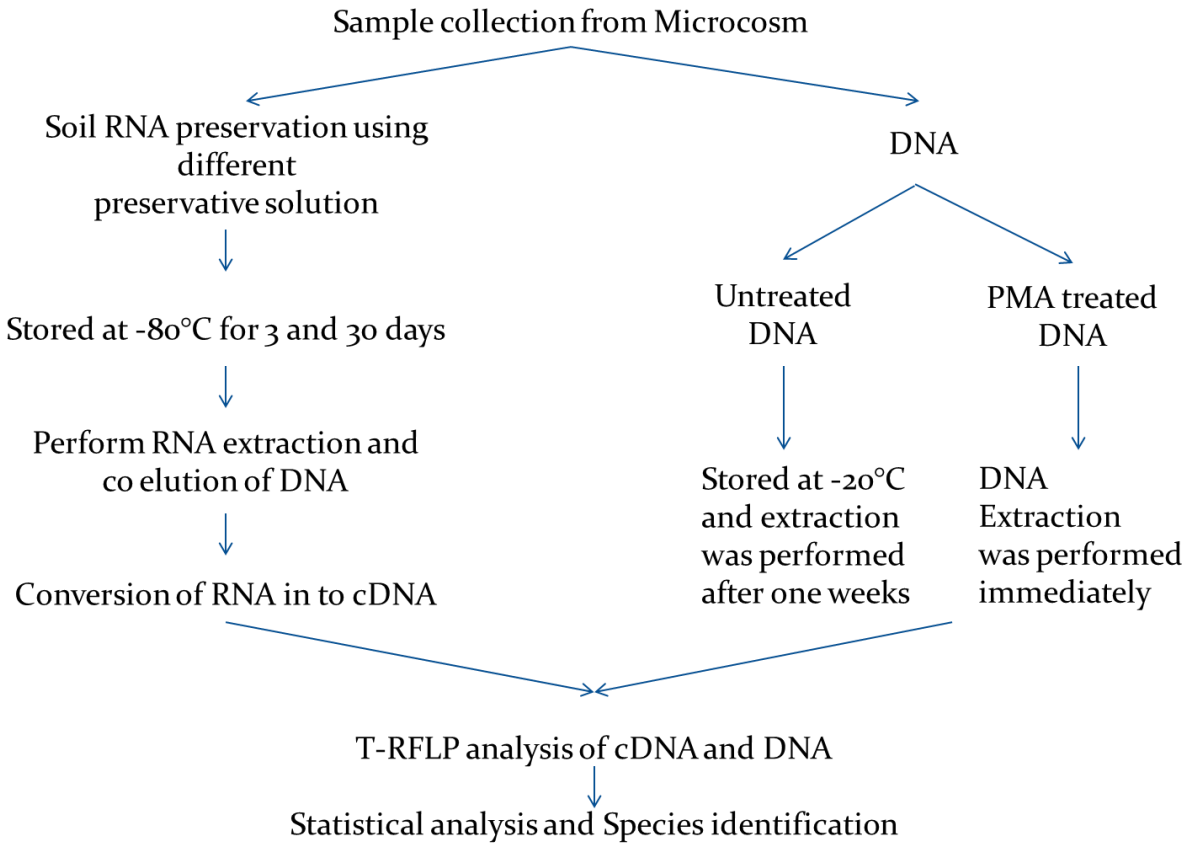

Figure 2.1: Flow chart describing the experimental design to compare DNA and RNA

2.2.4 PCR and TRFLP

The PCR of cDNA and DNA samples were performed using bacterial primers. The microbial 16S rRNA gene primer sets, bacterial forward, 8F: 5′-AGAGTTTGATCCTGGCTCAG-3′, bacterial reverse, 926r: 5′-CCGTCAATTCCTTTRAGTTT-3′ (Liu et al. 1997) were employed. The forward - primer was 6-5-Carboxyfluorescein 5-FAM labelled and synthesized together with the reverse primers by Applied Biosystems®. PCR reactions contain 3 µl of cDNA template, 1 µl of each primers and 15 µl of Hot Star Plus master mix solution (Qiagen, Toronto, Canada). PCR conditions were as previously described (Liu et al. 1997). Amplified PCR products were checked on a 1% agarose

gel, stained with Gel red and cleaned with the QIAquick PCR purification kit (Qiagen, Canada), following the manufacturer's instructions.

About 25 µl of the purified PCR product was digested with a fast digest protocol supplied with the DNA restriction enzymes, Hae III, Hha I and Msp I (Fermentas, Burlington, Canada), in separate reactions. From these, 2 µl was added to 9 µl of a solution made by adding 890 µl Hidi-formamide (Applied Biosystems, California USA) and 8 µl of Liz500 size standard (Applied Biosystems, California USA). Size calling was performed on 3310 ABI sequencer (Applied Biosystems, California USA) and fingerprints assembled on a Peak Scanner™ (Applied Biosystems, California USA). The peak scanner is free software widely used to view, edit and analyze the DNA fragment data from ABI sequencer. A cut off point for fragment sizes included in further analysis was between 50 and 500 bp, which were above the range for primer dimer formation and in the range of size standard. Terminal restriction fragment length polymorphism (TRFLP) analysis was done in triplicate for each sample.

2.2.5 Statistical analysis of TRFLP data

The output from the peak scanner was analyzed using T-align software (Smith et al. 2005). The T-align helps in the comparison of TRFLP data in the replicates of the sample and generates a consensus profile containing TRF only present in both replicates. The generated profile will be compared with other sample TRFLP consensus profiles to generate a matrix based on presence/absence of TRF. The output of the T-align can directly be used in the different statistical software. The presence/absence data was entered into the PAST software (Hammer et al. 2001) and different statistical analysis like Principal Component Analysis (PCA), Cluster analysis and Diversity Indices were performed.

The PCA is an ordination statistical tool widely used in TRFLP analysis. This tool has the ability to analyze large data sets by converting them into a smaller number of uncorrelated variables called components (Schütte et al. 2008). This property is essential in case of TRFLP analysis, where the datasets are larger in number. By converting the original data into new variables the tool has the ability to express the similarity and dissimilarity between the samples.

The cluster analysis is performed in order to identify the similarity between the samples using different similarity measures and to form groups between closely related samples. The Jaccard's index is the widely used method for presence/absence data. The grouping in the cluster analysis can be used to substantiate the results obtained through PCA.

The diversity indices like the Shannon index and Simpson index are widely applied in the field of Ecology but in recent times these indices are also used to analyze TRFLP data. The Shannon index (Shannon CE and Weaner W 1949) explains the diversity of a sample by counting the number of species (richness) and their relative abundance. In case of TRFLP, the number of TRF peaks will represent the richness and the peak area will represent the relative abundance. The sample with higher number of equally distributed microbial species will have higher diversity, so both richness and relative abundance have an impact on Shannon index. Even though the diversity indices are used in T-RFLP to explain the diversity of the samples, the Shannon index cannot reflect the genuine diversity of the sample (Blackwood CB 2007). In ecological studies, the diversity index of a geographical region is calculated based on physical evidence on the richness and abundance of plants/animals. Whereas in case of TRFLP peak area is used to calculate the abundance of the species but peak area is error prone. The numbers of PCR cycle, selection of restriction enzymes and capillary electrophoresis all have impact on peak area. Therefore in case of TRFLP the numbers provided by these indices will not be considered as actual diversity instead it will be relatively used to compare the samples. If the diversity index value is high for a sample it will be viewed as a sample with higher microbial diversity.

2.2.6 Species Identification

The species identification was performed using Phylogenetic Assignment Tool (PAT) (Kent. et al., 2003). In this web based tool the TRFLP data submitted by the user is compared with the predicted TRF data to identify the species. The software like MiCA (Microbial Community Analysis) (Shyu et al., 2007) have the ability to generate a TRF database based on the primer and enzyme information provided by user by in-silico digestion of 16S rRNA database. This TRF database can be used in PAT tool to compare original and predicted TRF and

to perform species identification. The PAT has the ability to handle TRF data from multiple enzyme digestion and narrow down the species. In this experiment data from three different enzyme digestions were used.

2.3 Results and Discussion

The T-RFLP analysis was performed using three different restriction enzymes (Hae lll, Hha l and Msp l) and digestion with each enzyme performed separately. Despite using three enzymes in restriction digestion to generate three separate TRFLP profiles for a single sample, the actual purpose of the three different TRFLP data was to use it in PAT species identification. The PAT requires TRF information from different enzyme digestion to narrow down the species names. In the case of statistical analysis, only the TRFLP profile generated from Hae lll enzyme was used. In case of statistical analysis TRF data from single restriction enzyme can provide significant information about the change in microbial community structure between the samples. Even though the TRFLP analysis was performed using replicate samples, the results will lack error bars/standard deviations. Replicate T-RFLP data were aligned using T-align (details in section 2.2.5) before being processed by PAST software, thus replicates are expressed as a single value.

2.3.1 Statistical analysis to determine the best RNA preservative solution

In this study, the PCA (Principal Component Analysis) and cluster analysis were used for two purposes, first to identify the best soil RNA preservation solution and second to study the similarity/difference between the RNA and DNA.

In the case of RNA samples three groups were observed. In the first group the control sample (CD1) and LifeGuard™ treated samples (LG3 and LG30) were found. The flash frozen samples (LN3 and LN30) and glycerol treated samples (GL3 and GL30) form the second and third group respectively (Fig 2.2). Even though the T-RFLP analysis was performed on RNAlater® treated samples (RL3 and RL30) these samples were not included in the statistical analysis because of lack of TRF. Similar to the result found in this study, a study by Rissanen et

al. 2010 had proved the inefficiency of RNAlater® to preserve RNA in soil. The RNAlater® having a high concentration of ammonium sulfate may cause precipitation and fixation of proteins and other organic compounds such as humic acids on nucleic acids (Rissanen et al. 2010). This directly affects the quantity and quality of RNA during extraction.

The control RNA being extracted immediately after sample collection will tend to have little to no RNA degradation. Therefore the control RNA will represent the actual microbial population found in the FFT sample. The similarities between treated/preserved RNA's and control RNA will be used as a proxy to determine the best preservation method. The LifeGuard™ treated samples seem to have higher similarity with control samples(Fig 2.2); this shows its efficiency in preserving RNA in complex environmental samples like oil sands tailings.

Here the cluster analysis of Jaccard's similarity index is used to confirm the results of PCA. The cluster analysis (Fig 2.3) was performed using presence/absence data. In the case of RNA two groups of clusters were formed. In this statistical analysis, even though a similarity was observed between liquid nitrogen treated samples and LifeGuard™ treated samples the major difference between the two methods are the inactivation of RNase. The LifeGuard™ solution has the ability to keep the RNase completely inactive during storage and homogenization of the samples. However, in the case of flash freezing, the reactivation of RNase is possible during homogenization and this may lead to degradation of RNA. Furthermore, the accessibility of liquid nitrogen in a field setting is also a significant challenge.

The Shannon index is the commonly used diversity index to explain the species richness and relative abundance of a sample, utilizing the peak area data of the TRF. The diversity index was used to compare different RNA samples. All of the samples appear to have high diversity, but with the aim being to simply compare the samples, the sample with the highest value will be considered the best preserved sample. From the comparison (Table 2.1) the 30 day lifeguard treated sample seemed to have the highest (value) diversity followed by control and 3 day flash frozen sample. The lowest diversity was observed in samples treated with glycerol. A study conducted by Sessitsch et al. 2002 proved the efficiency of glycerol in soil RNA preservation, but this same solution was found less effective in our samples. This proves the identification of

compatible RNA preservative solution should be the first step in the study of active microbial population. Even though the 3 day flash frozen sample showed a higher diversity a sharp decline in the diversity was observed after 30 days. On the other hand, the LifeGuard™ preservation saw a significant increase in diversity over the long-term storage period. This increase in total TRF after treating with lifeguard was also documented in our previous work (unpublished) but since the lifeguard solution is a patented solution the exact mechanism for this increase cannot be identified. This clearly proves the efficiency of the LifeGuard™ solution in preserving soil RNA both short-term and long-term.

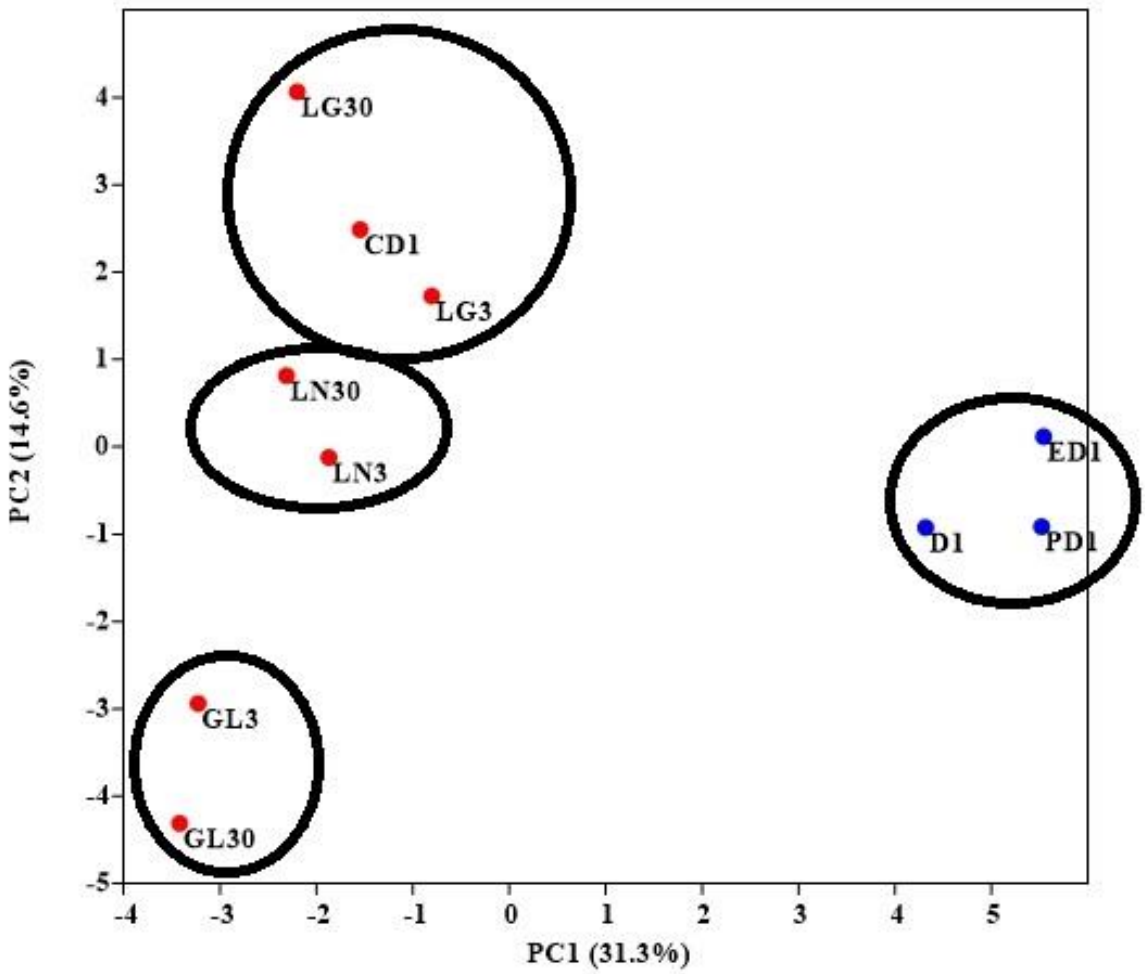

Figure 2.2: PCA based on Presence/Absence data of Bacterial 16s rRNA and 16s rDNA; Samples in Red and Blue represents RNA and DNA samples respectively. CD1 = RNA control, LG3 = LifeGuard™ treated after 3 d (RNA), LG30 = LifeGuard™ treated after 30 d (RNA), GL3 = Glycerol treated after 3 d (RNA), GL30 = Glycerol treated after 30 d (RNA), LN3 = Liquid nitrogen treated after 3 d (RNA), LN30 = Liquid nitrogen treated after 30 d (RNA). ED1 = Eluted DNA control, PD1 = PMA-DNA, D1 = untreated DNA.

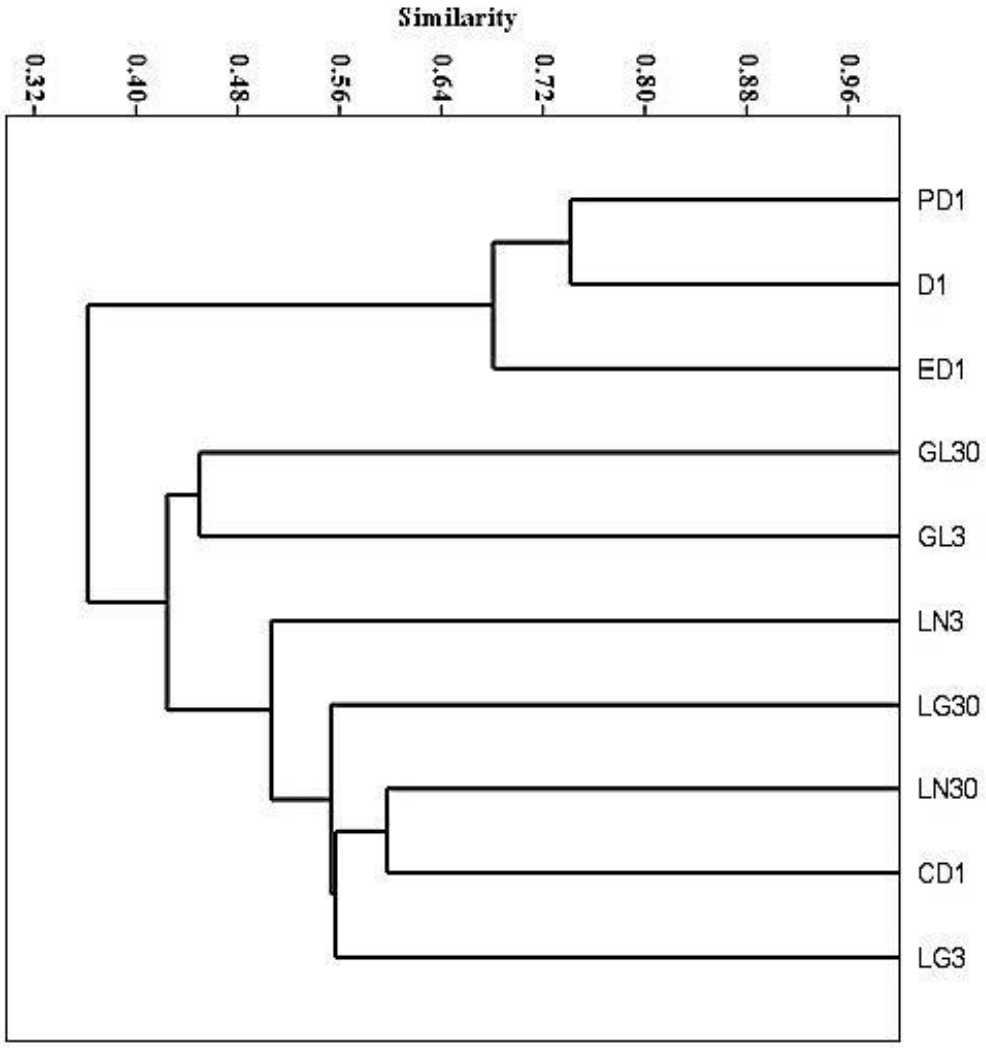

Figure 2.3: Cluster analysis - Jaccard's similarity based on Presence/Absence data of Bacterial 16s rRNA and 16s rDNA. CD1 = RNA control, LG3 = LifeGuard™ treated after 3 d (RNA), LG30 = LifeGuard™ treated after 30 d (RNA), GL3 = Glycerol treated after 3 d (RNA), GL30 = Glycerol treated after 30 d (RNA), LN3 = Liquid nitrogen treated after 3 d (RNA), LN30 = Liquid nitrogen treated after 30 d (RNA). ED1 = Eluted DNA control, PD1 = PMA-DNA, D1 = untreated DNA.

Table 2.1: Diversity index of RNA samples (CD1 = RNA control, LG3 = LifeGuard™ treated after 3 d (RNA), LG30 = LifeGuard™ treated after 30 d (RNA), GL3 = Glycerol treated after 3 d (RNA), GL30 = Glycerol treated after 30 d (RNA), LN3 = Liquid nitrogen treated after 3 d (RNA), LN30 = Liquid nitrogen treated after 30 d (RNA).

Samples	TRF	Shannon Index
CD1	123	4.316
LG3	111	4.228
LG30	135	4.483
GL3	89	4.139
GL30	85	4.130
LN3	116	4.304
LN30	99	4.212

2.3.2 Comparison of DNA, PMA-DNA and RNA

From the PCA (Fig 2.2) and cluster analysis (Fig 2.3) it is clear that the RNA characterization is quite distinct from that of DNA. Even though both RNA and DNA were isolated from same sample they tend to show a different microbial community structure which separates them in the PCA-plot and cluster analysis. A deeper understanding of this difference between DNA and RNA may help prove the importance of these nucleic acids in microbial ecology.

In this analysis three different types of DNA control were used, first the Eluted DNA control, then PMA untreated and PMA treated DNA. The second and third DNA control was isolated using Power soil DNA isolation kit (MO BIO Laboratories, Inc, California). Even though the Eluted DNA (EDNA) gave sufficient TRF for statistical analysis, the EDNA cannot be used for species identification. The EDNA was included in the experiment just to study the impact of the extraction method and sample quantity on DNA quality. Through statistical analysis it is clear that regardless of sample quantity and isolation method the EDNA, PMA-DNA and DNA have similarities. Unlike EDNA, the PMA untreated and PMA treated DNA

being isolated by the kit specifically designed for the DNA extraction it can be used for species identification and comparison with RNA.

The DNA is the most stable macromolecule and can persist in soil for a longer duration even after the death of the microorganism. By extracting DNA from soil samples extracellular DNA, DNA from both dead and live cells will be gained. Analyzing this DNA sample may lead to an overestimation of the microbial population. This issue might be solved by using Propidium Monoazide (PMA). Theoretically the analysis of PMA treated samples will give information only about the live cells. But its role in complex environmental samples is yet to be tested. Even though the PMA-DNA can give information about live cells, all live cells need not be active at all times. Thus the active microbial community of a sample can be studied using RNA. The comparison of DNA, PMA-DNA and RNA can give complete information about the microbial community structure of any sample.

From table 2.2, it is clear that the control RNA sample has higher amount of TRF followed by PMA-DNA and untreated DNA sample. But based on the previous description the untreated DNA sample should have a higher amount of TRF because it represents extracellular DNA, dead cells and live cells. The TRF of PMA-DNA should be a subset of untreated DNA because it represents only the live cells. The TRF of control RNA sample should be a subset of PMA-DNA because it represents only the active cells. Our hypothesis about the DNA, PMA-DNA and RNA was disproved, so further analysis was performed to find the factors responsible for these changes.

The table 2.3 compares the untreated DNA to PMA-DNA based on their unique TRF and shared TRF. From this data it is clear that the TRF of untreated DNA is principally a subset of PMA-DNA because most of the TRF of untreated DNA is shared with PMA-DNA, while only the PMA-DNA has unique TRF. This loss of TRF in the untreated DNA sample may be due to storage, extraction, PCR and T-RFLP analysis. In the experiment the only difference between the untreated DNA and PMA-DNA was their storage. The untreated DNA samples were stored at -20°C for a period of one week and extraction was performed, whereas in case of PMA-DNA after sample collection, the samples were treated with PMA and the extraction was performed

immediately. This difference in storage between the DNA sample and the PMA - DNA sample was not deliberate but an unexpected one. The PMA-DNA extraction was performed immediately because the purpose of PMA was to study live cells and therefore extraction without storage is crucial. Some previous studies (Lauber et al. 2010, Larson et al. 2009) reported that the different storage methods have no effect on DNA quality and quantity, but this is not true for all types of samples (Lee et al. 2007). From the experiment, it is clear that the samples like oil sands tailings with high concentration of clay and other organic compounds may have an impact on the concentration of DNA during extraction (Ogram et al. 1988, Cai et al., 2005). The storage of complex environmental samples provides an increased opportunity for clay like particles to bind to DNA and inhibit its extraction. The untreated DNA lost some TRF and did not reflect the actual sample diversity compared to PMA-DNA, therefore for the purpose of comparison with control RNA, the data of PMA-DNA was used.

The Table 2.4 compares PMA-DNA and RNA based on their unique TRF and shared TRF. From the data it is clear that both DNA and RNA have higher numbers of both unique and shared TRF. The hypothesis states that the TRF of RNA is merely a subset of PMA-DNA, but through the analysis it was proved wrong. The presence of unique TRF in RNA was also been documented in some previous work (Mengoni et al. 2005, Nogales et al. 2001). Unique TRF in DNA may be found because of the presence of inactive live cells in the sample. The actual reason for the difference between RNA and DNA are not well documented. Further analysis has to be performed to understand this difference between the two macromolecules. This clearly proves that in the case of complex environmental samples like oil sands tailings both DNA and RNA should be studied to understand the actual microbial community structure.

2.3.3 Species Identification

In order to compare PMA-DNA and RNA based on microbial population the species identification was performed using a phylogenetic assignment tool (PAT). In PAT the T-RFLP data from three different restriction enzymes is used for species identification. In the table 2.5 the number of species included will be less compared to the total number of TRF, because of the presence of unidentified species. Based on the analysis it is clear, as stated before some

microbial species were observed using both DNA and RNA, whereas some were only detected using one of the two nucleic acids. This study focused on SRB communities as they are considered the leading contributor in the sulfur cycle. On comparing PMA-DNA and extracted RNA, a greater diversity of SRB was detected in PMA-DNA compared to RNA signatures (Table 2.5). The sample used for my analysis was collected from a 20 week old microcosm and as per another experiment, during this time the sulfate reduction rate was declining in this sample. This shows that the SRB were alive at the time of sample collection but not metabolically active to be identified by RNA. This demonstrates the importance of RNA in microbial ecology both in terms of identifying only metabolically active species and giving information about species which are not identified by DNA.

Table 2.2: Comparison of DNA, PMA-DNA and RNA based on their Total TRF

Samples	Number of TRF
Untreated DNA	85
PMA DNA	110
Control RNA	123

Table 2.3: Comparison of untreated DNA and PMA-DNA depicting their unique and shared TRF

Samples	Unique TRF	Common TRF
Untreated DNA	2	83
PMA-DNA	27	

Table 2.4: Comparison of Control RNA and DNA depicting their unique and shared TRF

Samples	Unique TRF	Common TRF
PMA-DNA	39	71
Control RNA	52	

Table 2.5: Comparison of Control RNA and PMA-DNA based on bacterial species identified using Phylogenetic Assignment Tool (PAT)

S.no	Bacterial Species	
	PMA-DNA control	RNA control
	Sulfate Reducing Bacteria:	Sulfate Reducing Bacteria:
1	*Desulfobacter* sp	*Desulfovibrio* sp
2	*Desulfobulbus* sp	
3	*Desulforhopalus* sp	
4	*Desulfotignum* sp	
5	*Desulfotomaculum* sp	
6	*Desulfovibrio* sp	
7	*Desulfuromonas* sp	
	Other common Microbes:	Other common Microbes:
1	*Achromatium* sp	*Acidovorax* sp
2	*Acidovorax* sp	*Anaeromyxobacter* sp
3	*Anaeromyxobacter* sp	*Arthrobacter* sp
4	*Arthrobacter* sp	*Caldanaerobacter* sp
5	*Azospira* sp	*Chlorobium* sp
6	*Burkholderia* sp	*Denitrovibrio* sp
7	*Calditerrivibrio* sp	*Geobacillus* sp
8	*Chromatiales* sp	*Oxalobacteraceae* sp
9	*Cupriavidus* sp	*Prosthecochloris* sp
10	*Dechloromonas* sp	*Ralstonia* sp
11	*Delftia* sp	*Rhodanobacter* sp
12	*Geobacter* sp	*Rhodococcus* sp
13	*Methylosinus* sp	*Rhodoferax* sp

14	*Polaromonas* sp	*Rhodopseudomonas* sp
15	*Ralstonia* sp	*Sphingomonas* sp
16	*Rhodococcus* sp	*Holophaga* sp
17	*Rhodoferax* sp	*Nitrospirae* sp
18	*Rhodopseudomonas* sp	*Sulfurihydrogenibium* sp
19	*Sideroxydans* sp	
20	*Thiothrix* sp	
21	*Verrucomicrobiales* sp	

2.4 Conclusions

In this study T-RFLP of 16s rRNA/cDNA was successfully used to identify the best preservation method for microbial RNA in the oil sands tailings sample. The statistical analysis of T-RFLP data have shown a higher similarity between lifeguards treated samples and control sample. Thus we conclude that the LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc, California) can be used for short-term and long-term preservation of soil microbial RNA. This finding is crucial as the LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc, California) can be successfully employed for field sample collection and preservation of oil sands tailings. The RNAlater® solution specifically designed for tissue samples seems to be incompatible with the tailings sample. The glycerol preservation method on comparison with lifeguard method seems to be less efficient in preserving soil RNA both short and long-term. Even though the flash freezing was effective for short-term storage, the RNA quality was compromised after long-term storage.

Even though the goal in comparing untreated DNA sample and PMA treated DNA samples was to find the effectiveness of PMA in identifying live microbial population in FFT, the difference in storage of the samples revealed a different but significant result. The PMA-DNA extracted immediately after sample collection had a higher number of TRF compared to untreated DNA, which was stored at -20°C for a week before extraction. Therefore in order to reflect the actual microbial community structure of complex environmental samples the DNA extraction has to be done immediately after sample collection.

The PMA-DNA and RNA were compared based on total number of TRF and through species identification to detect their significance in microbial ecology. Even though theoretically, the TRF from RNA is merely a subset of PMA-DNA, through the analysis it was determined that both PMA-DNA and RNA have shared and unique TRF. Therefore in any sample, in order to get a clear understanding of the live microbial community structure, both PMA-DNA and RNA has to be utilized.

Chapter 3 - Evaluation of Microbial community structure in FFT using T-RFLP

3.1 Introduction

Biogeochemical processes are driven by microorganisms in all types of environments. As stated in General Introduction (Chapter 1), successful conversion of oil sands tailings ponds to wetlands requires an understanding of the biogeochemical processes taking place within the tailings. To reclaim oil sands tailings ponds as wetlands, the FFT material, which forms the basic substrate of these wetlands, has to be harmless to the aquatic organisms. To determine the potential effects these tailings may have on aquatic organisms, long-term assessment of the biogeochemical processes occurring in the FFT has to be considered. These assessments have to be performed in different tailings ponds operated by different industries to understand the similarity and difference between the ponds, which is crucial for pond management. In this study the samples were collected from STP (South Tailings Pond) operated by Suncor Energy Inc. The results obtained from STP were compared with our previous study (Chi Fru et al. 2013 and Chen et.al 2013) performed on WIP (West in Pit) operated by Syncrude Canada Ltd to establish the relationship between the ponds (Fig 3.1).

Being an essential nutrient for most of the aquatic organisms dissolved oxygen is responsible for the successful development of wetland ecosystems. Therefore the study on sediment oxygen demand is of primary importance. A study conducted by Gelda et al 1995 has shown the role of methane, ammonia and sulfides for sediment oxygen demand in Lake system, with sulfides being the largest contributor to SOD. Though some environments will have less impact from sulfides, oil sands tailings material has a significant sulfate reducing bacterial community, therefore playing a significant role on SOD (Ramos-Padrón et al. 2011, Holowenko et al. 2000). Study by Chen et.al 2013 has proved the higher activity of sulfate reducing bacteria and in turn production of higher amount of sulfides. Even though the sulfate reducing bacteria was successfully used in hydrocarbon degradation and inhibition of methanogenesis (Holowenko et al. 2000, Fedorak et al. 2002), the hydrogen sulfide produced during these processes are toxic (Smith and Oseid 1971) and highly reductive in nature. It is these properties of hydrogen sulfide that negatively impact the establishment of functional wetlands from tailings ponds. Therefore

the study on sulfate reduction and other associated chemical processes are crucial in order to develop a reclamation procedure.

In this study laboratory microcosm experiments are used to understand the chemical and biological processes taking place in fluid fine tailings. The study by Chi Fru et al. 2013 showed that the microbial community structure in laboratory microcosms represent the community structure in the actual tailings ponds. In the study, the chemical analysis was performed by Reid et al (unpublished) using sensitive micro sensors and pore water extraction techniques to understand the chemical cycles taking place in FFT. This thesis will concentrate on biological analysis, the T-RFLP method was used to understand the microbial community structure of FFT (Fig 3.2). The T-RFLP method being a PCR based method is widely used to demonstrate a shift in microbial community structure and species identification (Schütte et al. 2008, Kent et al. 2003). The 16s rDNA (16s rRNA gene) of both Bacteria and Archaea were analyzed by T-RFLP throughout the study period (20 weeks) to understand the change in the community structure over time. Different statistical methods were used to analyze the T-RFLP data, to establish the similarities/differences between the samples collected from microcosms at different time intervals, atmospheric conditions and depths. Species identification was performed to track the SRB population in the FFT samples. The information obtained through statistical analysis and species identification will be used in the future to validate the chemical data.

3.2 Materials and Methods

3.2.1 Sample collection

The fluid fine tailings (FFT) and oil sands processed water (OSPW) were obtained from South Tailings Ponds (STP) operated by Suncor Energy Inc, located in the Athabasca region, Alberta. The FFT and OSPW were shipped to our laboratory in 20 liter buckets and these samples upon arrival were separated into two batches. One batch was stored at 4°C. The other batch sent to McMaster Nuclear Reactor (MNR), Hamilton for gamma irradiation treatment. The FFT and OSPW were gamma irradiated at 28KGY over 24 hour's leading to complete elimination of biological activity. These samples were used as abiotic controls.

3.2.2 Experimental Design

The evolution of the biogeochemical processes within the FFT was studied for a period of 20 weeks through laboratory microcosm experiments. The experiments were performed under both oxic and anoxic conditions (Fig 3.3). The abiotic (gamma irradiated) sample was also included in the experiment as a control. The sample collections from the microcosms were performed after 4, 8 and 20 weeks. For each sampling period two biotic and abiotic microcosms (replicates) were maintained under each atmospheric condition. The microcosms used in this study were clear plastic tubes with a flat base and an independent PVC cap, designed by the University of Windsor technical support center. The microcosms were sterilized using 95% ethanol before introducing the FFT. To all the microcosms, approximately 800 g of FFT and 400 g of OSPW was added. The FFT and OSPW were thoroughly mixed using a power portable drill with a sterilized Teflon-coated stirring paddle. Anaerobic microcosms were given an airtight PVC lid using closed-cell weatherstripping. The headspaces were flushed with ultra-pure nitrogen and placed in the anaerobic chamber to maintain anaerobic conditions. All the microcosms were maintained under dark environment at room temperature.

3.2.3 Microcosm sample collection

The FFT samples were semi-solid in nature and were collected through the disposable sterilized micropipette tip using 5 ml micropipette. The sample collection was performed at water-sediment interface and at the bottom of the microcosm. Immediately after sample collection, the samples were treated with PMA and DNA extractions were performed.

3.2.4 PMA treatment and DNA extraction

PMA™ (Propidium Monoazide) dye, 20 mM in H2O (Biotium, Inc) was used in the experiment (Nocker et al. 2006). 100 µM of PMA was added to 0.25g sample and mixed thoroughly. The samples were incubated in the dark for 5 minutes at room temperature. After incubation the sample tubes were placed on the ice and were exposed to light for 5 minutes using 600 w halogen light source. Immediately after PMA treatment the DNA extractions were

performed using PowerSoil® DNA Isolation Kit (Mo Bio, Laboratories Inc, California) following manufacturer's instruction, with the extracted DNA being stored at -20°C. The extractions were performed in triplicate and were pooled together before performing further experiments.

Fig 3.1: Satellite image showing the location of STP and WIP

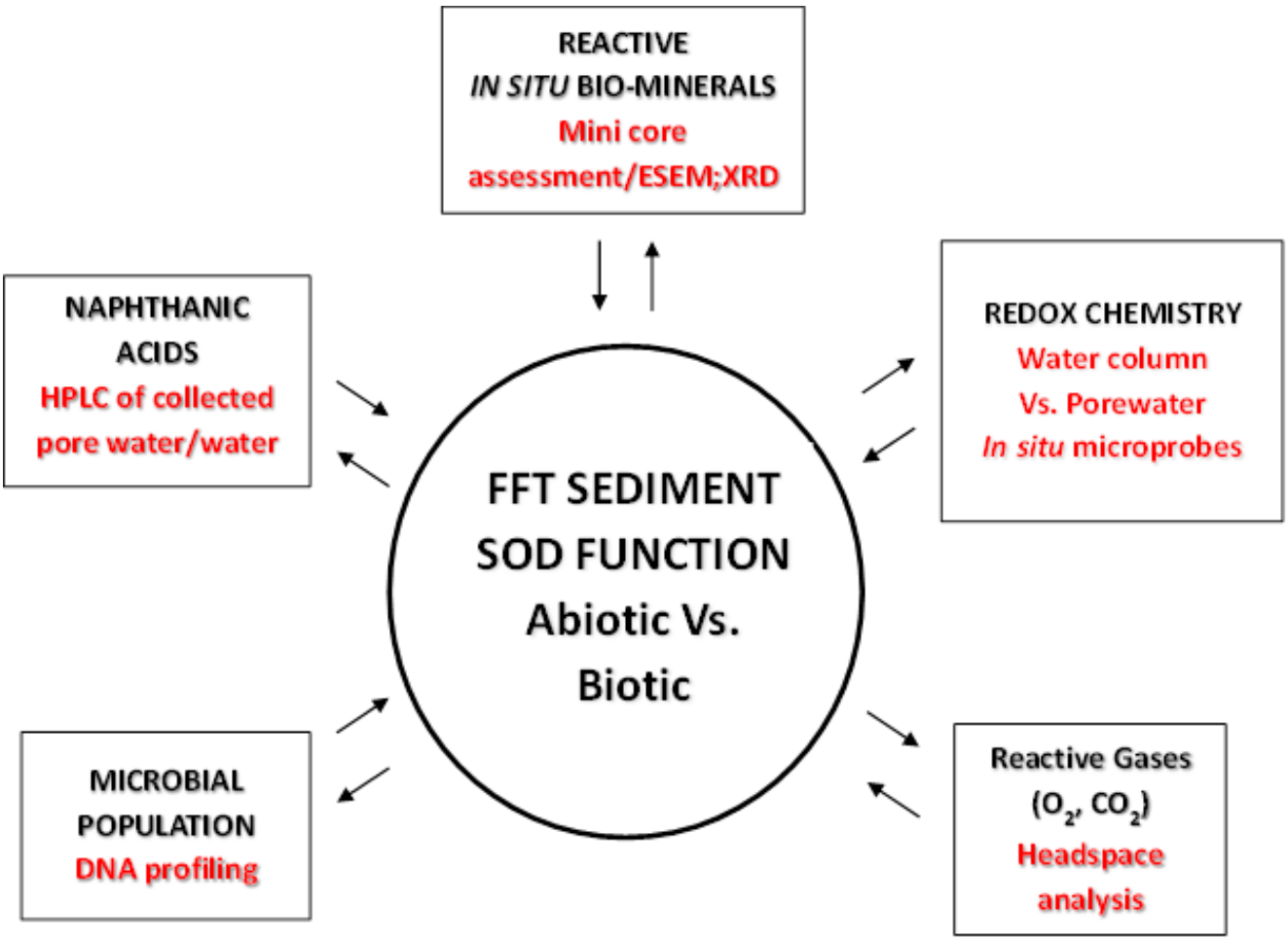

Figure 3.2: Conceptual diagram depicting different work involved in characterization of FFT

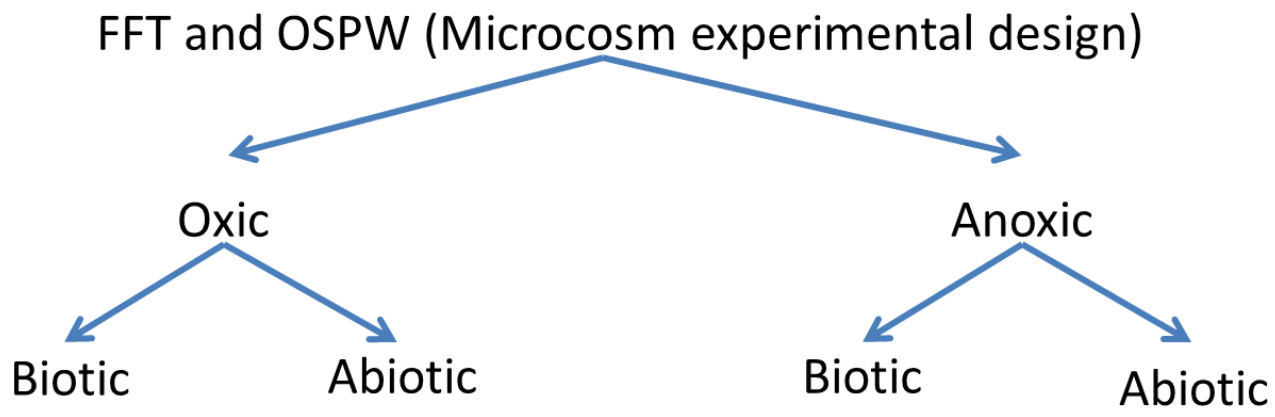

Fig 3.3: Experimental Design

3.2.5 PCR and TRFLP

The PCR of PMA-DNA samples was performed using both bacterial and archaeal primers. The 16S rRNA gene primer sets, bacterial forward, 8F: 5′-AGAGTTTGATCCTGGCTCAG-3′, bacterial reverse, 926r: 5′-CCGTCAATTCCTTTRAGTTT-3′ (Liu et al. 1997). Archaeal forward, 109f:5′-ACKGCTCAGTAACACGT-3′. Archaeal reverse, A934b: 5′-GTGCTCCCCCGCCAATTCCT-3′ (Großkopfet al. 1998) was used. Forward-primers were 6-5-Carboxyfluorescein (5-FAM, bacteria) and NED™ (archaea) labelled and synthesized together with the reverse primers by Applied Biosystems. PCR reactions contain 3µl of DNA template, 1µl of each primers and 15µl of Hot Star Plus master mix solution (Qiagen, Toronto, Canada). PCR conditions were as previously described (Liu et al. 1997; Großkopf et al. 1998). Amplified PCR products were checked on a 1 % agarose gel, stained with Gel red and cleaned with the QIAquick PCR purification kit (Qiagen, Canada), following the manufacturer's instructions.

About 25 µl of the purified PCR product was digested with a fast digest protocol supplied with the DNA restriction enzymes, Hae III, Hha I and Msp I (Fermentas, Burlington, Canada), in separate reactions. From these, 2 µl was added to 9 µl of a solution made by adding 890 µl Hidi-formamide (Applied Biosystems, California USA) and 8 µl of Liz500 size standard (Applied Biosystems, California USA). Size calling was performed on 3310 ABI sequencer (Applied Biosystems, California USA) and fingerprints assembled on a Peak Scanner™ (Applied Biosystems, California USA). The peak scanner is free software widely used to view, edit and analyze the DNA fragment data from ABI sequencer. A cut off point for fragment sizes included in further analysis was between 50 and 500 bp, which were above the range for primer dimer formation and in the range of size standard. Terminal restriction fragment length polymorphism (TRFLP) analysis was done in triplicate for each sample.

3.2.6 Statistical analysis of TRFLP data

The output from the peak scanner was analyzed using T-align software (Smith et al. 2005). The T-align helps in the comparison of TRFLP data in the replicates of the sample and generates a consensus profile containing TRF only present in both replicates. The generated

profile will be compared with other sample TRFLP consensus profiles to generate a matrix based on presence/absence of TRF. The output of the T-align can directly be used in the different statistical software. The presence/absence data was entered into the PAST software (Hammer et al. 2001) and different statistical analysis like Principal Component Analysis (PCA), Cluster analysis and Diversity Indices were performed.

The PCA is an ordination statistical tool widely used in TRFLP analysis. This tool has the ability to analyze large data sets by converting them into a smaller number of uncorrelated variables called components (Schütte et al. 2008). This property is essential in case of TRFLP analysis, where the datasets are larger in number. By converting the original data into new variables the tool has the ability to express the similarity and dissimilarity between the samples.

The cluster analysis is performed in order to identify the similarity between the samples using different similarity measures and to form group between closely related samples. The Jaccard's index is the widely used method for presence/absence data. The grouping in the cluster analysis can be used to substantiate the results obtained through PCA.

The diversity indices like the Shannon index and Simpson index are widely applied in the field of Ecology but in recent times these indices are also used to analyze TRFLP data. The Shannon index (Shannon CE and Weaner W 1949) explains the diversity of a sample by counting the number of species (richness) and their relative abundance. In case of TRFLP, the number of TRF peaks will represent the richness and the peak area will represent the relative abundance. The sample with higher number of equally distributed microbial species will have higher diversity, so both richness and relative abundance have an impact on Shannon index. Even though the diversity indices are used in T-RFLP to explain the diversity of the samples, the Shannon index cannot reflect the genuine diversity of the sample (Blackwood CB 2007). In case ecological studies, the diversity index of a geographical region is calculated based on physical evidence on the richness and abundance of plants/animals. Whereas in case of TRFLP peak area is used to calculate the abundance of the species but peak area is error prone. The numbers of PCR cycle, selection of restriction enzymes and capillary electrophoresis all have impact on peak area. Therefore in case of TRFLP the numbers provided by these indices will not be considered

as actual diversity instead it will be relatively used to compare the samples. If the diversity index value is high for a sample it will be viewed as a sample with higher microbial diversity.

3.2.7 Species Identification

The species identification was performed using Phylogenetic Assignment Tool (PAT) (Kent et al. 2003). In this web based tool the TRFLP data submitted by the user will be compared with the predicted TRF data to identify the species. The software like MiCA (Microbial Community Analysis) (Shyu et al. 2007) have the ability to generate a TRF database based on the primer and enzyme information provided by user by in-silico digestion of 16S rRNA database. This TRF database can be used in PAT tool to compare original and predicted TRF and to perform species identification. The PAT has the ability to handle TRF data from multiple enzyme digestion and narrow down the species. In this experiment data from three different enzyme digestions were used.

3.3Results and discussion

3.3.1 Statistical analysis to understand the temporal and spatial changes of Bacterial community structure

In the study the PCA (Figure 3.4) was included to track the change in microbial community structure for a period of 20 weeks in oil sands tailings. The PCA was performed based on presence/absence T-RFLP data of 16s rDNA of bacterial species. Even though the archaeal 16s rDNA was included in the TRFLP analyses the TRF data were not included in any statistical analysis, this because the archaeal DNA in the sample was identified only during week 20. Therefore to identify the difference between the samples bacterial PMA-DNA was used. The first principal component contributes to around 21.4% of variation and the second component contributes to nearly 18.4% of variation. From the figure three groups can be distinguished.

The week 4 samples regardless of their atmospheric condition and depth have similar microbial population and were all grouped together. Whereas this trend changed during 8^{th} week,

where a higher dissimilarity was observed between samples collected at different depth. The 8th week samples collected from the bottom layer of the microcosm were grouped together irrespective of their atmospheric conditions. Whereas the samples collected from the upper layer of oxic and anoxic microcosm were placed separately in the PCA. This difference between the two samples may be because of the development in the activity of oxidizing bacteria and other aerobic bacteria in the oxic microcosm. During 8th week a huge difference was observed between the samples collected at different depth. This difference was primarily because of the increased microbial Population at the water-sediment interface. This higher microbial population in the upper layer was also documented in the previous study (Chi Fru et al. 2013, Penner & Foght 2010, Ramos-Padrón et al. 2011). The Sulfate reducing Bacteria might be responsible for this increased microbial population. The presence of higher amount of sulfate can directly correlates to the high activity of SRB. It has been reported that the tailing ponds have a higher concentration of sulfate in the water-sediment interface and it declines as the depth increases (Ramos-Padrón et al. 2011). In case of 20th week all the samples were placed separately from each other this may be because of the development of new groups of bacterial species after the decline of most common dominant species especially sulfate reducing bacteria. In our previous study (Chi Fru et al. 2013), it was reported that the microbial population in samples collected from aerobic and anaerobic microcosms were similar. In contrary to this report, in this study we found differences in the bacterial population in the samples based on atmospheric conditions. This shows irrespective of being covered by overlying water column, the FFT receives dissolved oxygen and it shapes the bacterial community of the sample. But this information can be validated only by comparing with the chemical data of the microcosm samples.

The cluster analysis by Jaccard's similarity index (Figure 3.5) was performed based on presence/absence data. The samples seem to be clustered into three groups and these groups are formed mainly based on time of sample collection. The subgrouping in the cluster reflects the similarity among the aerobic and anaerobic samples and dissimilarity between the samples collected at different depth. The highest similarity of about 66-76% was observed between 4th week samples and lowest degree of similarity was observed in 20th week samples and it was around 30-46%. This higher dissimilarity between the samples collected during the 20th week,

clearly proves the development of different microbial community over time regardless of atmospheric condition and depth.

From the Shannon index (table 3.1) it is clear that at 4 weeks the bacterial population was homogeneously distributed throughout the microcosm, whereas at 8 weeks a noticeable shift in the population between the interface and the bulk portions of the microcosm were observed. The shift in microbial diversity in FFT, matrix has also been observed by others (Chi Fru et al. 2013, Fedorak et al. 2002, MacKinnon 1989) where the higher diversity was often associated with the formation of sulfide-rich zones. In my investigation sulfidic zones were not observed directly at this time point, but the presence of different sulfate reducing organisms was detected. Samples collected at 20 weeks continue to show a diverse bacterial population throughout the microcosm.

From the T-RFLP analysis (Fig 3.6) it is clear that irrespective of atmospheric condition and depth all the microcosms were dominated by bacterial community and the archaeal population was detected only during the 20^{th} week sampling. This initial dominance of bacterial species and the later emergence of archaeal species were reported in our previous study (Chi Fru et al. 2013). The SRB and Methanogens both compete for same electron source and in the presence of higher amount of sulfate the sulfate reducing bacteria dominates the environment resulting in a suppression of the activity of Methanogens (Lovley et al, 1982). Therefore only after the decline of SRB community will the emergence of methanogens become dominant and therefore detectable. The delay in the establishment of Archaeal population can also be related to its selection of electron source. Most of Archaeal species will utilize simpler carbon substrates derived from complex carbon molecules via other bacterial species. This shows irrespective of sample collection sites (Tailings Ponds) the shift in the microbial population follows a similar trend. This information is crucial for pond management because a similar reclamation approach can be developed for different tailings ponds.

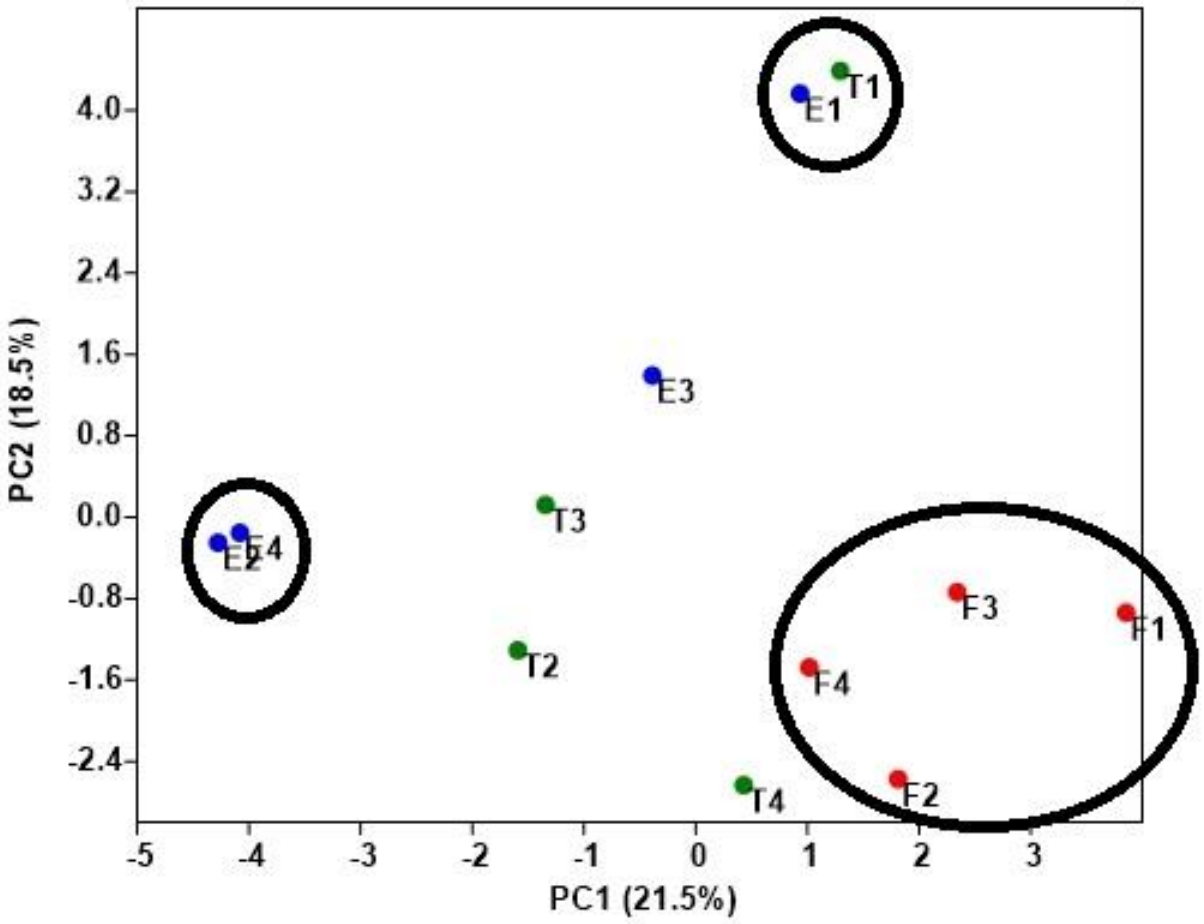

Figure 3.4: PCA of FFT samples based on Presence/Absence data of Bacterial 16s rDNA (PMA-DNA). Where the F1 & F2 represents 4th week oxic upper and bottom layer respectively, F3 & F4 represents 4th week anoxic upper and bottom layer respectively, E1 & E2 represents 8th week oxic upper and bottom layer respectively, E3 & E4 represents 8th week anoxic upper and bottom layer respectively, T1 & T2 represents 20th week oxic upper and bottom layer respectively, T3 & T4 represents 20th week anoxic upper and bottom layer respectively

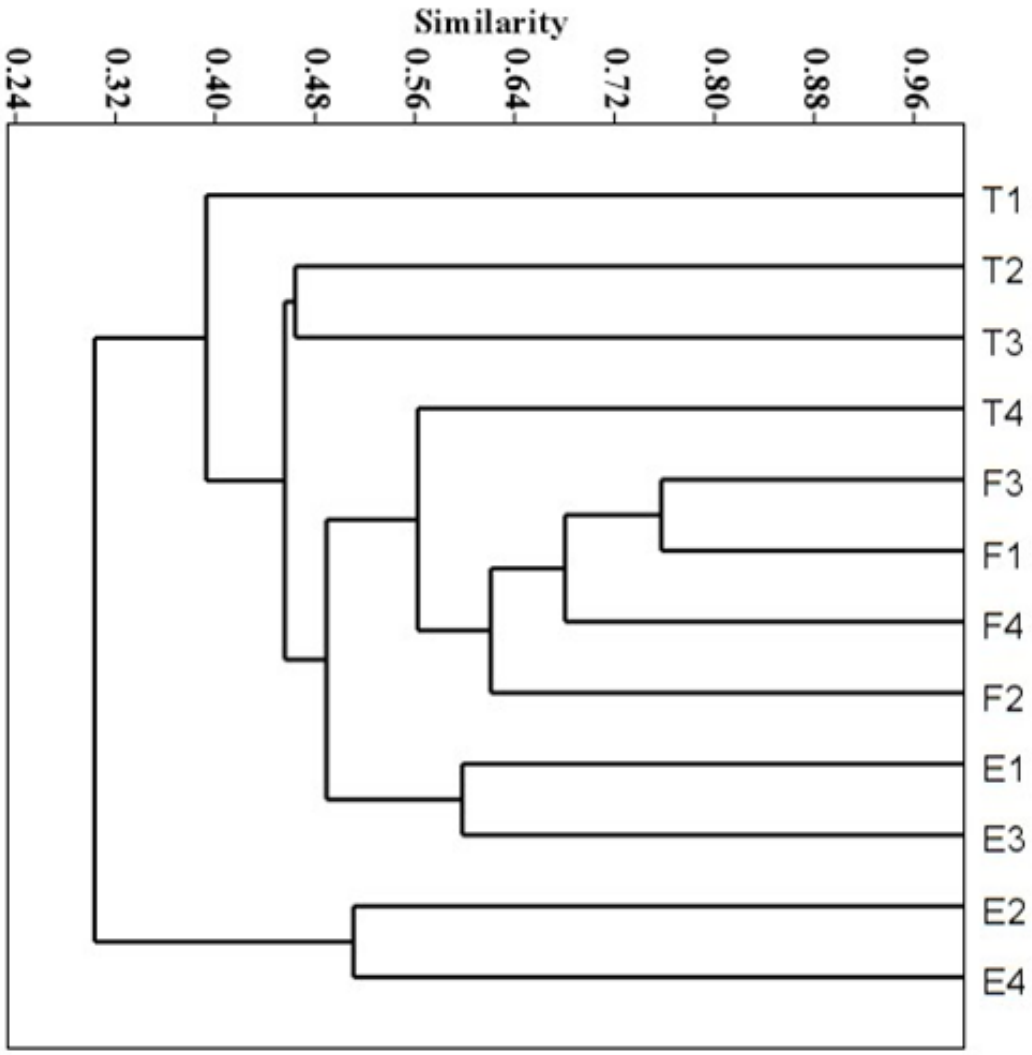

Figure 3.5: Cluster analysis of FFT samples - Jaccard's similarity based on Presence/Absence data of Bacterial 16s rDNA (PMA-DNA). Where the F1 & F2 represents 4th week oxic upper and bottom layer respectively, F3 & F4 represents 4th week anoxic upper and bottom layer respectively, E1 & E2 represents 8th week oxic upper and bottom layer respectively, E3 & E4 represents 8th week anoxic upper and bottom layer respectively, T1 & T2 represents 20th week oxic upper and bottom layer respectively, T3 & T4 represents 20th week anoxic upper and bottom layer respectively.

Table 3.1: Diversity Index of FFT samples based on relative abundance (peak area) of Bacterial 16s rDNA (PMA-DNA). Where the F1 & F2 represents 4th week oxic upper and bottom layer respectively, F3 & F4 represents 4th week anoxic upper and bottom layer respectively, E1 & E2 represents 8th week oxic upper and bottom layer respectively, E3 & E4 represents 8th week anoxic upper and bottom layer respectively, T1 & T2 represents 20th week oxic upper and bottom layer respectively, T3 & T4 represents 20th week anoxic upper and bottom layer respectively.

Samples	Shannon index
F1	4.064
F2	3.838
F3	3.963
F4	3.812
E1	4.036
E2	2.886
E3	3.924
E4	3.195
T1	3.73
T2	3.896
T3	3.926
T4	3.84

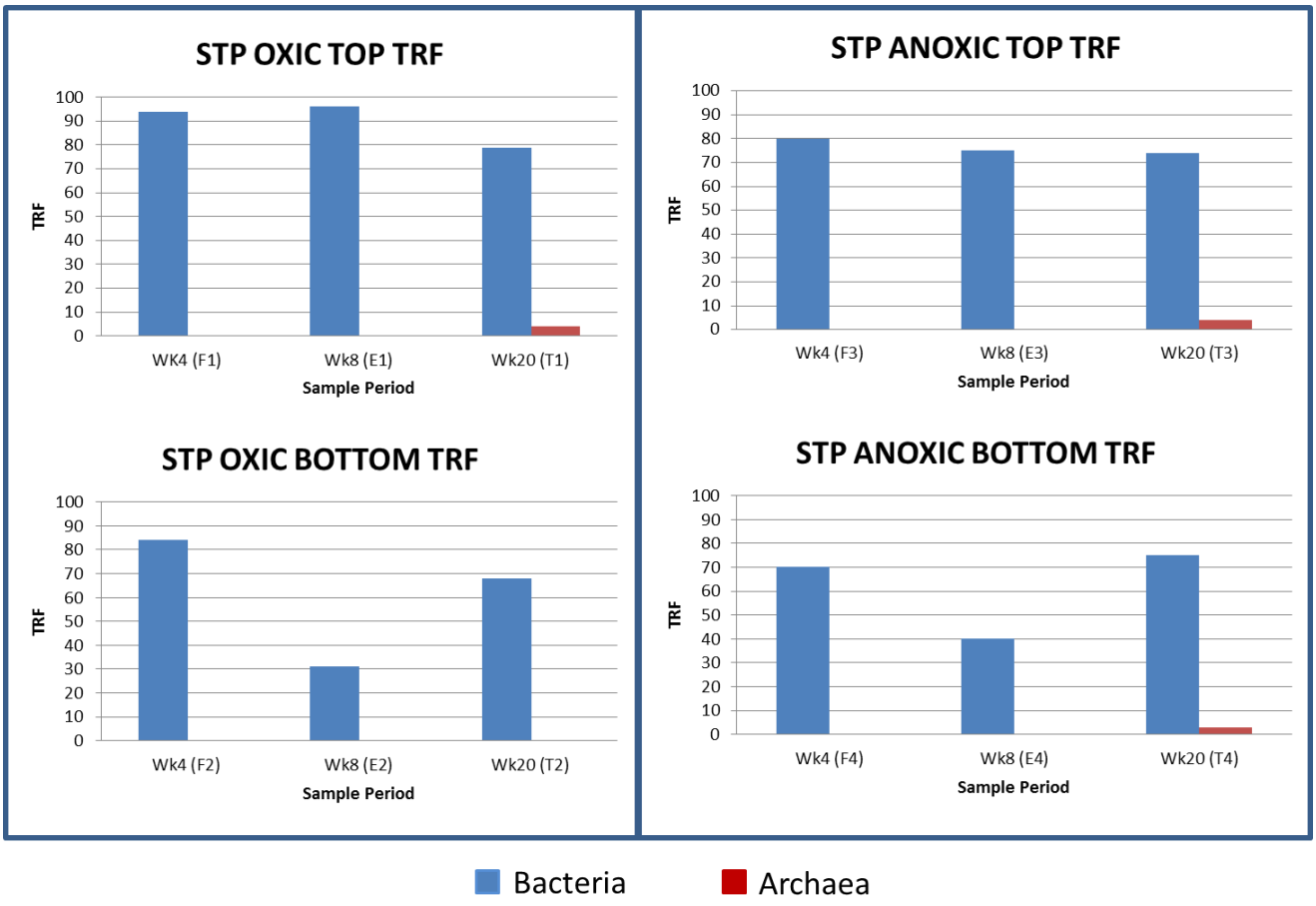

Fig 3.6: Number of Bacterial and Archaeal TRF (Terminal Restriction Fragments) of FFT samples. Where the F1 & F2 represents 4th week oxic upper and bottom layer respectively, F3 & F4 represents 4th week anoxic upper and bottom layer respectively, E1 & E2 represents 8th week oxic upper and bottom layer respectively, E3 & E4 represents 8th week anoxic upper and bottom layer respectively, T1 & T2 represents 20th week oxic upper and bottom layer respectively, T3 & T4 represents 20th week anoxic upper and bottom layer respectively.

3.3.2 Species Identification

In order to compare samples based on microbial population the species identification was performed using a phylogenetic assignment tool (PAT). In PAT the T-RFLP data from three different restriction enzymes will be used for species identification. Bacterial species present in the samples are listed in tables 3.2, 3.3, 3.4 & 3.5. Even though different species were identified using PAT, the primary purpose of this study was to track the presence of SRB. The information about other species will be used in the future to compare with sequencing results.

In the case of oxic microcosm, irrespective of depth different species of SRB were dominating the microcosm during week 4 and 8 of the study. This changed over time and during the 20^{th} week there was a decline in the activity of SRB and no SRB species were detected in the upper layer of the microcosm but some SRB activity was still observed in the lower layer of the microcosm. This loss in SRB activity over time was also documented in our previous study (Chen et al. 2013) and this decline in sulfate reduction is crucial for pond management.

The prolonged activity of SRB leads to higher production of sulfides. These sulfides forms metal sulfides and get precipitated but in case of insufficient amount of metals in the tailings, these sulfides reach the overlying water column and consumes oxygen. By consuming oxygen in the process of oxidizing to sulfate/sulfur they may cause sediment oxygen demand, which will impact aquatic life. But from the study it is clear that the sulfate reduction driven by bacteria is a self-limiting process and therefore will have less impact on the quality of the overlying water column over time.

In case of anoxic microcosm, regardless of depth the SRB population seems to be low during week 4 but as the system matures (week 20) higher number of SRB species was identified. This result contradicts with the result obtained from oxic microcosm where during the same time no/less SRB species were detected. This demonstrates the role of dissolved oxygen in shaping the microbial community in FFT. But in order to get a clear picture about this difference the chemical information about the microcosms are crucial. Even though in this thesis, the microbial data of FFT for 20 weeks were included the actual experiment runs for 52 weeks.

Therefore after completion of the experiment both microbial and chemical data will be analyzed to determine the cause for the difference between oxic and anoxic microcosm. Even though both aerobic and anaerobic microcosms were studied to understand changes in microbial community structure over time, the higher number of TRF was observed only in aerobic microcosms. The oil tailings ponds being an open system the aerobic microcosms will represent the tailings ponds more closely compared to anoxic microcosms. Therefore in this chapter further discussions and conclusions will be made based on oxic system.

Through species identification it is clear that the FFT harbors a wide range of biogeochemical processes. The PAT T-RFLP analysis identified bacterial species responsible for Nitrogen cycle, Iron cycle and Methane cycle. The nitrogen cycle is a predominant process in FFT and some of the following species were identified responsible for the process, *Alicycliphilus* sp, *Azospira* sp, *Arthrobacter* sp, *Anaeromyxobacter* sp, *Anabaena* sp, *Bergeriella* sp, *Calditerrivibrio* sp and *Nitrosomonas* sp. Iron is an important nutrient for all microorganisms. The hydrogen sulfide produced during sulfate reduction will react with iron to form iron sulfides and will get precipitated. This process may cause iron depletion and thereby affect the growth of organisms in FFT. Therefore the study of iron cycle in FFT is of pivotal importance. Some of the following species were involved in iron cycling, *Albidiferax* Sp, *Acidovorax* sp and *Sideroxydans* sp. For the successful conversion of tailings ponds into wetlands the FFT material should to less toxic, the bioremediation of the FFT is the only way to reduce the toxicity. The utilization of indigenous microorganisms is considered to be the best strategy for bioremediation. Through the PAT different bacterial species involved in hydrocarbon and heavy metal degradation were identified. The *Burkholderia* sp, *Ralstonia* sp, *Geobacter* sp, *Polaromonas* sp, *Pseudomonas* sp, *Comamonas* sp and *Cupriavidus* sp are some of the species involved in remediation in FFT. This information on the microorganisms and their role in the biochemical cycles will be utilized in the future to develop molecular tools to track microbial group specific genes and in turn understand their activity in FFT. The data on microbial and chemical activity will be an asset for successful establishment of wetland ecosystems on FFT.

Table 3.2: Comparing bacterial species present in the upper layer of the oxic microcosm (Where the F1, E1 & T1 represents 4th, 8th & 20th week oxic upper layer respectively)

S.no	Bacterial Species		
	F1	E1	T1
	Microbes involved in Sulfur Cycle:	Microbes involved in Sulfur Cycle:	Microbes involved in Sulfur Cycle:
1	*Achromatium* sp	*Achromatium* sp	*Achromatium* sp
2	*Chloroflexus* sp	*Chlorobium* sp	*Thiomonas* sp
3	*Desulfobotulus* sp	*Desulfatibacillum* sp	
4	*Desulfobulbus* sp	*Desulfobacter* sp	
5	*Desulfofrigus* sp	*Desulfobulbus* sp	
6	*Desulfotalea* sp	*Desulfotomaculum* sp	
7	*Desulfovibrio* sp	*Desulfovibrio* sp	
8	*Desulfuromonas* sp	*Desulfurivibrio* sp	
9	*Sulfitobacter* sp	*Desulfuromonas* sp	
	Other Common Microbes	Other Common Microbes	Other Common Microbes
1	*Acidovorax* sp	*Acidovorax* sp	*Agrobacterium* sp
2	*Alcaligenes* sp	*Agrobacterium* sp	*Alcaligenes* sp
3	*Alicycliphilus* sp	*Alicycliphilus* sp	*Anaeromyxobacter* sp
4	*Anaeromyxobacter* sp	*Alicyclobacillus* sp	*Arthrobacter* sp
5	*Ancylobacter* sp	*Anaeromyxobacter* sp	*Brevibacillus* sp
6	*Burkholderia* sp	*Ancylobacter* sp	*Burkholderia* sp
7	*Calyptogena* sp	*Arthrobacter* sp	*Cupriavidus* sp
8	*Dechloromonas* sp	*Azospira* sp	*Dechloromonas* sp
9	*Delftia* sp	*Bacillus* sp	*Delftia* sp
10	*Diaphorobacter* sp	*Brevibacillus* sp	*Denitrobacter* sp
11	*Gallionella* sp	*Burkholderia* sp	*Diaphorobacter* sp
12	*Geobacter* sp	*Dechloromonas* sp	*Geobacter* sp

13	*Lysinibacillus* sp	*Deferribacterales* sp	*Methylophilus* sp
14	*Methylovorus* sp	*Delftia* sp	*Oxalobacteraceae* sp
15	*Polaromonas* sp	*Diaphorobacter* sp	*Polaromonas* sp
16	*Pseudomonas* sp	*Geobacter* sp	*Pseudomonas* sp
17	*Rhodoferax* sp	*Methylobacterium* sp	*Ralstonia* sp
18		*Polaromonas* sp	*Rhodobacter* sp
19		*Pseudomonas* sp	*Rhodoferax* sp
20		*Ralstonia* sp	*Rhodomicrobium* sp
21		*Rhodanobacter* sp	*Sideroxydans* sp
22		*Rhodoferax* sp	
23		*Sideroxydans* sp	
24		*Streptomyces* sp	
25		*Tistrella* sp	

Table 3.3: Comparing bacterial species on lower layer of the oxic microcosm (Where the F2, E2 & T2 represents 4th, 8th & 20th week oxic lower layer respectively)

S.no	Bacterial Species		
	F2	E2	T2
	Microbes involved in Sulfur Cycle:	Microbes involved in Sulfur Cycle:	Microbes involved in Sulfur Cycle:
1	*Achromatium* sp	*Achromatium* sp	*Achromatium* sp
2	*Desulfatibacillum* sp	*Desulfobacter* sp	*Desulfatibacillum* sp
3	*Desulfobacter* sp	*Desulfobulbus* sp	*Desulfobacter* sp
4	*Desulfobotulus* sp	*Desulfovibrio* sp	*Desulfotalea* sp
5	*Desulfobulbus* sp	*Desulfurivibrio* sp	*Desulfovibrio* sp
6	*Desulfovibrio* sp	*Desulfuromonas* sp	*Desulfuromonas* sp
7	*Desulfuromonas* sp		
	Other Common Microbes	Other Common Microbes	Other Common Microbes
8	*Acidovorax* sp	*Achromobacter* sp	*Acidovorax* sp
9	*Agrobacterium* sp	*Acidovorax* sp	*Alcaligenes* sp
10	*Alicyclobacillus* sp	*Alicycliphilus* sp	*Alicycliphilus* sp
11	*Anaeromyxobacter* sp	*Arthrobacter* sp	*Azospira* sp
12	*Arthrobacter* sp	*Azospira* sp	*Brevibacillus* sp
13	*Azospira* sp	*Brevibacillus* sp	*Burkholderia* sp
14	*Bacillus* sp	*Burkholderia* sp	*Chlorobium* sp
15	*Brevibacillus* sp	*Delftia* sp	*Chloroflexi* sp
16	*Burkholderia* sp	*Diaphorobacter* sp	*Dechloromonas* sp
17	*Dechloromonas* sp	*Geobacter* sp	*Delftia* sp
18	*Delftia* sp	*Polaromonas* sp	*Denitrovibrio* sp
19	*Denitrovibrio* sp		*Diaphorobacter* sp
20	*Diaphorobacter* sp		*Geobacter* sp

21	*Geobacter* sp		*Mariprofundus* sp
22	*Methylobacillus* sp		*Methylophilus* sp
23	*Methylobacterium* sp		*Methylovorus* sp
24	*Methylovorus* sp		*Nitrosomonas* sp
25	*Polaromonas* sp		*Pelomonas* sp
26	*Pseudomonas* sp		*Polaromonas* sp
27	*Rhodoferax* sp		*Pseudomonas* sp
28	*Sideroxydans* sp		*Ralstonia* sp
29	*Sinobacter* sp		*Rhodoferax* sp
30	*Streptomyces* sp		*Sideroxydans* sp
31			*Sphingomonas* sp
32			*Variovorax* sp

Table 3.4: Comparing bacterial species on upper layer of the anoxic microcosm (Where the F3, E3 & T3 represents 4th, 8th & 20th week anoxic upper layer respectively)

S.no	Bacterial Species		
	F3	E3	T3
	Microbes involved in Sulfur Cycle:	Microbes involved in Sulfur Cycle:	Microbes involved in Sulfur Cycle:
1	*Achromatium* sp	*Achromatium* sp	*Achromatium* sp
2	*Chloroflexus* sp	*Chlorobium* sp	*Chlorobium* sp
3	*Desulfobulbus* sp	*Chloroflexus* sp	*Chloroflexus* sp
4	*Desulfovibrio* sp	*Desulfatibacillum* sp	*Desulfatibacillum* sp
5	*Desulfurispirillum* sp	*Desulfobacter* sp	*Desulfobacter* sp
6		*Desulfobulbus* sp	*Desulfobulbus* sp
7		*Desulfurivibrio* sp	*Desulfofrigus* sp
8		*Desulfuromonas* sp	*Desulfotalea* sp
9			*Desulfurivibrio* sp
10			*Desulfuromonas* sp
11			*Prosthecochloris* sp
12			*Sulfitobacter* sp
13			*Thiomonas* sp
	Other Common Microbes	Other Common Microbes	Other Common Microbes
1	*Achromobacter* sp	*Achromobacter* sp	*Acidovorax* sp
2	*Acidovorax* sp	*Acidovorax* sp	*Acinetobacter* sp
3	*Alcaligenes* sp	*Acinetobacter* sp	*Agrobacterium* sp
4	*Alicycliphilus* sp	*Albidiferax* Sp	*Alcaligenes* sp
5	*Alicyclobacillus* sp	*Alcaligenes* sp	*Alicycliphilus* sp
6	*Anaeromyxobacter* sp	*Alicycliphilus* sp	*Anabaena* sp
7	*Arthrobacter* sp	*Alicyclobacillus* sp	*Anaeromyxobacter* sp

8	*Bacillus* sp	*Anabaena* sp	*Ancylobacter* sp
9	*Burkholderia* sp	*Anaeromyxobacter* sp	*Arthrobacter* sp
10	*Comamonas* sp	*Ancylobacter* sp	*Azospira* sp
11	*Dechloromonas* sp	*Arthrobacter* sp	*Bacillus* sp
12	*Delftia* sp	*Azospira* sp	*Bergeriella* sp
13	*Diaphorobacter* sp	*Azospira* sp	*Brevibacillus* sp
14	*Geobacter* sp	*Bacillus* sp	*Burkholderia* sp
15	*Methylophilus* sp	*Brevibacillus* sp	*Calditerrivibrio* sp
16	*Methyloversatilis* sp	*Burkholderia* sp	*Chromobacterium* sp
17	*Methylovorus* sp	*Cupriavidus* sp	*Comamonas* sp
18	*Microbacterium* sp	*Dechloromonas* sp	*Cupriavidus* sp
19	*Polaromonas* sp	*Delftia* sp	*Dechloromonas* sp
20	*Pseudomonas* sp	*Diaphorobacter* sp	*Deinococcus* sp
21	*Ralstonia* sp	*Dyella* sp	*Delftia* sp
22	*Rhodoferax* sp	*Geobacter* sp	*Diaphorobacter* sp
23	*Variovorax* sp	*Ilyobacter* sp	*Geobacter* sp
24		*Kinetoplastibacterium* sp	*Methylobacterium* sp
25		*Leptothrix* sp	*Methylomonas* sp
26		*Methylocystis* sp	*Methylophilus* sp
27		*Methylomonas* sp	*Methyloversatilis* sp
28		*Methylophilus* sp	*Methylovorus* sp
29		*Mitsuaria* sp	*Polaromonas* sp
30		*Nitrosomonas* sp	*Polynucleobacter* sp
31		*Polaromonas* sp	*Pseudomonas* sp
32		*Polynucleobacter* sp	*Ralstonia* sp
33		*Ralstonia* sp	*Rhodobacter* sp
34		*Ramlibacter* sp	*Rhodoferax* sp
35		*Rhodoferax* sp	*Rhodomicrobium* sp
36		*Spirillum* sp	*Rhodopila* sp
37		*Tistrella* sp	*Rhodopseudomonas* sp
38			*Spirillum* sp

Table 3.5: Comparing bacterial species on lower layer of the anoxic microcosm (Where the F4, E4 & T4 represents 4th, 8th & 20th week anoxic lower layer respectively)

S.no	Bacterial Species		
	F4	E4	T4
	Microbes involved in Sulfur Cycle:	Microbes involved in Sulfur Cycle:	Microbes involved in Sulfur Cycle:
1	*Achromatium* sp	*Achromatium* sp	*Achromatium* sp
2	*Chloroflexus* sp	*Chloroflexus* sp	*Chlorobium* sp
3	*Desulfobulbus* sp	*Desulfobulbus* sp	*Chloroflexus* sp
4	*Desulfurivibrio* sp	*Desulfofrigus* sp	*Desulfatiferula* sp
5		*Desulfovibrio* sp	*Desulfobotulus* sp
6		*Desulfurivibrio* sp	*Desulfobulbus* sp
7		*Desulfuromonas* sp	*Desulfocurvus* sp
8			*Desulfofrigus* sp
9			*Desulfoluna* sp
10			*Desulfotalea* sp
11			*Desulfurivibrio* sp
	Other Common Microbes	Other Common Microbes	Other Common Microbes
1	*Achromobacter* sp	*Acidovorax* sp	*Acidovorax* sp
2	*Acidovorax* sp	*Albidiferax* sp	*Alcaligenes* sp
3	*Alcaligenes* sp	*Alcaligenes* sp	*Alicycliphilus* sp
4	*Alicycliphilus* sp	*Alicycliphilus* sp	*Alicyclobacillus* sp
5	*Alicyclobacillus* sp	*Alicyclobacillus* sp	*Anabaena* sp
6	*Anaeromyxobacter* sp	*Anabaena* sp	*Anaeromyxobacter* sp
7	*Arthrobacter* sp	*Arthrobacter* sp	*Anaeroplasma* sp
8	*Azospira* sp	*Azospira* sp	*Ancylobacter* sp
9	*Bacillus* sp	*Bacillus* sp	*Arthrobacter* sp

10	*Bergeriella* sp	*Brevibacillus* sp	*Azospira* sp
11	*Brevibacillus* sp	*Burkholderia* sp	*Bacillus* sp
12	*Burkholderia* sp	*Comamonas* sp	*Brevibacillus* sp
13	*Comamonas* sp	*Dechloromonas* sp	*Burkholderia* sp
14	*Dechloromonas* sp	*Delftia* sp	*Calditerrivibrio* sp
15	*Delftia* sp	*Diaphorobacter* sp	*Calyptogena* sp
16	*Diaphorobacter* sp	*Geobacter* sp	*Chromobacterium* sp
17	*Geobacter* sp	*Methylophilus* sp	*Comamonas* sp
18	*Methylocystis* sp	*Methyloversatilis* sp	*Dechloromonas* sp
19	*Methylophilus* sp	*Mitsuaria* sp	*Delftia* sp
20	*Methyloversatilis* sp	*Pseudomonas* sp	*Diaphorobacter* sp
21	*Methylovorus* sp	*Ralstonia* sp	*Dietzia* sp
22	*Mitsuaria* sp	*Rhodoferax* sp	*Geobacter* sp
23	*Nitrosomonas* sp	*Sideroxydans* sp	*Mariprofundus* sp
24	*Polaromonas* sp		*Methylophilus* sp
25	*Pseudomonas* sp		*Methyloversatilis* sp
26	*Ralstonia* sp		*Methylovorus* sp
27	*Rhodoferax* sp		*Polaromonas* sp
28	*Sideroxydans* sp		*Polynucleobacter* sp
29	*Spirillum* sp		*Ralstonia* sp
30	*Variovorax* sp		*Rhodoferax* sp
31			*Rhodopseudomonas* sp
32			*Roseiflexus* sp
33			*Sideroxydans* sp
34			*Sphingomonas* sp
35			*Spirillum* sp
36			*Variovorax* sp

3.4 Conclusions

This study was performed in order to understand the change in microbial community structure in FFT over time. The microorganisms are the driving force of biogeochemical cycles. Therefore the study of microbial population can provide information on chemical activities. Microorganisms are considered to be principle protagonists in many biogeochemical environments responsible for cycling Sulfur, Nitrogen and Carbon. In this study T-RFLP analysis was used to discern the bacterial and archaeal 16s rDNA. The analysis showed irrespective of atmospheric condition and depth within the microcosm chambers key stages of microbial development. Initially the FFT matrix is dominated by bacteria which are eventually displaced by the emergence of archaeal populations after 20 weeks and increases during the later stages of the experiment. This difference in growth between bacteria and archaea could be due to increased toxicity with the increased evolution of HS production during sulfate reduction, which will suppress many bacterial species thus creating a niche for archaeal groups. The availability of electron source will also decide the emergence of microbial population.

By monitoring the onset of biodiversity and the immergence of key microbial species the information could be coupled to track the onset of sulfate reduction and methanogenesis taking place in FFT. The hydrogen sulfide produced during sulfate reduction being the key component of sediment oxygen demand in wetlands. The tracking of SRB population is of pivotal importance. The statistical analysis and species identification have been performed to understand change in bacterial population especially SRB population. The analysis showed a higher SRB population at the sediment - water interface during week 8 but it started to decline and only a few species were detected during week 20. This shows the sulfate reduction being a short-term process and has less impact on the quality of the overlying water column over time. This information is highly significant to establish functional wetlands on FFT.

CHAPTER 4 - Summary and Future Work

4.1 Summary

The RNA is synthesized by microorganisms only while they are active and involved in biochemical processes. Therefore the RNA analysis is the key to study the both microbial and chemical activity in any environment. Even though the RNA is useful to track biochemical process, the Ribose sugar in RNA makes it prone to chemical degradation and thereby makes it unstable in nature. Therefore the preservation of RNA will always be the primary step in any experiment. Oil sands tailings are a complex matrix and the chances of RNA degradation in such samples are very high. In this thesis different RNA preservation methods were tested to find the method most suitable for FFT material. Through T-RFLP analysis of 16s rRNA/cDNA the LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc, California) was found to be the best preservative method for FFT. The ability of the LifeGuard™ Soil Preservation Solution to inactivate RNase is the key to the successful preservation. Only a few studies (Sessitsch et al. 2002 and Foti et al., 2008) were done in case of microbial RNA preservation in environmental samples and this was the first study on RNA preservation in FFT samples. The presence of clay and humic substances make the FFT a complex environmental sample and therefore the RNA preservation method developed through this study can be effectively used for other complex samples.

Even though other preservative methods like glycerol or RNAlater® (Qiagen) were successfully used in preserving different soil and sediment samples, they were found to be incompatible with FFT. Even though RNAlater® has the property of RNase inactivation, in case of FFT it tends to release high amounts of humic acid and leads to co precipitation of RNA and in turn affects its extraction. Despite being a higher similarity was established between lifeguard samples and flash frozen samples, the main purpose of producing an RNA preservation protocol is to use it in field studies. In case of oil sands tailings ponds because of safety requirements it is practically impossible to perform flash freezing for large amount of samples. Whereas in case of LifeGuard™ Soil Preservation Solution, it is easily accessible and easy to handle under field conditions.

The comparison between DNA, PMA-DNA and RNA were performed based on T-RFLP data and through the comparison significant results in the field of microbial ecology was obtained. Unexpected storage of FFT sample for one week at -20°C and subsequent DNA extraction and analysis have proved that the complex environmental samples like FFT on storage may lose some amount of DNA. The clay, humic acid and other components present in the FFT during storage will bind to DNA and inhibit their release during extraction. This may lead to the underestimation of the microbial population in FFT. Therefore in order to get uncompromised data on microbial population, the DNA extraction has to be performed immediately after sample collection.

The role of PMA-DNA and RNA in microbial ecology was studied through T-RFLP analysis. Through the analysis it was understood that even though the PMA-DNA and RNA was isolated from same sample both have a higher number of shared and unique TRF. The unique TRF represents the complexity of microbial population in FFT. The FFT being nutrient rich in nature can harbor wide range of microorganisms and DNA alone cannot represent all the communities. The RNA along with providing information on microbial activity can give a clear picture on microbial communities in FFT.

Our project aims to compare biogeochemical data of samples (FFT) collected from different tailings ponds (Chapter 3). Therefore the microbial data generated through T-RFLP analysis of PMA-DNA in this thesis were compared with our previous work (Chi Fru et al., 2013, Chen et al., 2013) to establish the relationship between the two tailings ponds. Through the comparison, I found that irrespective of the sample collection sites, all the samples (FFT) were initially dominated by bacterial populations and later by archaeal populations. The bacterial populations especially SRB in the presence of sufficient nutrients are known to out-compete archaeal populations and thereby inhibit their growth. The archaeal population is known to utilize simple carbon sources and therefore the breakdown of complex carbon sources by bacteria is important for their establishment. The above mentioned reasons could be responsible for the later dominance of archaeal populations.

The information collected in this project will be used to understand the biogeochemical processes taking place in the FFT and its impact on sediment oxygen demand influencing the overlying water column. As stated before, sulfate reduction and methanogenesis are some of the processes responsible for sediment oxygen demand influencing the overlying water column. Through the analysis, it was found that the population of SRB was high during the initial period of sampling, but it declined in the later stages of analysis. It proves that the sulfate reduction process will be a short-term process and therefore have less impact on water quality.

4.2 Future Work

This study is first of its kind to develop an RNA preservation protocol for FFT samples and to apply PMA-DNA and RNA to study microbial community structure in FFT. Even though LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc, California) was found to be the best preservative method for FFT, the method need to be optimized before applying for field studies. In case oil sands tailings, during sample collection the researchers are denied access because of safety requirements. The sample collection takes a few hours and therefore RNA preservation is delayed this may lead to lose in active microbial population. This difference in sample collection, preservation and subsequent loss of microorganisms has to be studied. The FFT sample from laboratory microcosms will be used as a proxy to understand this phenomenon. The sample collection and subsequent RNA preservation will be performed at different hours (i.e. 1, 3 & 5 hours) and microbial community structure of the samples will analyze through T-RFLP. The results obtained will be used to optimize RNA preservation and molecular methods for field studies.

The RNA protocol developed will be applied to study active microbial population of FFT in laboratory microcosms. After successful completion of Laboratory microcosm studies, the Field mesocosm experiments will be performed in order to understand the change in microbial community structure in natural conditions. The mesocosms will be maintained in the environment similar to the oil sands tailings ponds and the sampling will be performed for a period of three years. This study will provide a better understanding about the biogeochemical process in tailing ponds.

Even though my results suggest the long-term preservation of RNA using LifeGuard™ Soil Preservation Solution and short-term preservation of RNA using liquid nitrogen, it is contrary to the previous literature. The manufacturer of LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc, California) suggests the usage of the solution for a storage-period of 30 days. In contrast the flash freezing is well known to keep the nucleic acids intact for longer periods. Therefore in future experiments, the comparison between the lifeguard method and the flash freezing method will be performed over a longer storage-period.

Even though the Propidium Monoazide (PMA) treated DNA was used in the study of microbial community structure, the efficiency of PMA to remove external DNA from FFT have not been proved in this study because of the difference in the storage of PMA treated DNA and untreated DNA. This similar experiment to compare PMA-DNA and DNA will be repeated with both the DNA's are being extracted immediately after sample collection. By treating both DNA in similar condition, the efficiency of PMA in FFT can be studied. This experiment is crucial in order to study live microbial population in FFT samples.

Even though the T-RFLP data was used to perform species identification this is not a most preferred method in the field of microbial ecology. The nucleic acid sequencing is the most successful method for species identification. In the project the Ion Torrent Next-Generation Sequencing will be used to analyze both DNA and cDNA/RNA as the method is considered to be cost effective and more precise. The Ion Torrent Next-Generation sequencing finds its application in the field of Metagenomics and Metatranscriptomics. In case of Metagenomics, the 16s rDNA and 16s rRNA extracted directly from environmental samples (FFT) will be analyzed to determine microbial community structure. Whereas in case of Metatranscriptomics, the mRNA from the FFT samples will sequenced to determine the specific gene function. The mRNA is synthesized while the microbes are actively participating in the biogeochemical process. Therefore the Metatranscriptomics can be used to establish a direct link between monitored chemical activity and the expression of microbial activity. In the project, Q PCR analysis of mRNA of dsr gene and mcr gene will also be performed. This analysis will provide information on the activity of sulfate reducing bacteria and methanogens respectively. At any

given time, if the mRNA concentration of dsr gene is high it directly implies the higher activity of SRB and in turn higher sulfate reduction. Therefore the mRNA information can be used to validate the chemical data

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